new directions in
microbial methane oxidation

een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen, Wiskunde en Informatica

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Ashna

contents

samenvatting

nieuwe ontdekkingen in microbiële methaanoxidatie

Methaan (CH_4), ook wel aardgas of moerasgas genoemd, is in 1778 ontdekt door de Italiaanse natuurkundige Alessandro Volta. In 1900 toonde de Nederlandse microbioloog, Nicolaas Söhngen, aan dat methaan geproduceerd kan worden door micro-organismen. Zes jaar later ontdekte dezelfde microbioloog dat er ook bacteriën bestaan die methaan omzetten met behulp van zuurstof.

Microbiologen hebben zich lang afgevraagd of bacteriën methaan ook konden omzetten zonder zuurstof. In de jaren 70 van de twintigste eeuw zijn de eerste aanwijzingen gevonden dat methaan met behulp van sulfaat kan worden omgezet. De sulfaat-afhankelijke omzetting van methaan vindt plaats op de bodem van de oceaan waar zowel methaan als sulfaat in overvloed aanwezig zijn. Het bewijs dat micro-organismen in staat waren dit proces uit te voeren kwam pas veel later: eind jaren 90. Recent onderzoek heeft aangetoond dat niet alleen micro-organismen, maar ook planten in staat zijn methaan te produceren. Microbiologen zijn dus al heel lang geïnteresseerd in hoe de biologische methaancyclus in elkaar zit.

Het onderzoek dat in dit proefschrift beschreven wordt, richtte zich op de ontdekking van nieuwe micro-organismen die methaan als brandstof gebruiken in zowel zuurstofrijke and zuurstofarme milieu's. Het begrijpen van de microbiële methaanomzetting is belangrijk omdat methaan een sterk broeikasgas is, wel 20 keer sterker dan koolstofdioxide. Op plaatsen waar veel organisch materiaal, maar geen zuurstof is, leven micro-organismen die methaan produceren. Dit gebeurt bijvoorbeeld in de darmen van termieten, in de magen van herkauwers en in veengebieden.

Uit onderzoek naar de emissie van methaan in veengebieden bleek dat er relatief weinig methaan vrijkwam en dat microben waarschijnlijk verantwoordelijk waren voor de omzetting van methaan. De eerste hoofdstukken van dit proefschrift zijn dan ook gericht op methaanoxidatie met behulp van zuurstof in veengebieden. Methaanoxidatie is de door micro-organismen gekatalyseerde omzetting van methaan en zuurstof naar koolstofdioxide en water. Al gauw bleek dat in veengebieden bacteriën verantwoordelijk waren voor de omzetting van methaan in koolstofdioxide en dat daardoor de emissie van methaan naar de atmosfeer laag bleef.

Er werd ontdekt dat de onder water levende veenmossen voor hun groei afhankelijk waren van de koolstofdioxide die door de micro-organismen werd geproduceerd. De methaanetende bacteriën, ook wel methanotrofe bacteriën genoemd, leven op en zelfs in deze veenmossen en zetten methaan en zuurstof om in water en koolstofdioxide. Na een korte algemene inleiding (hoofdstuk één), beschrijft hoofdstuk twee van dit proefschrift de symbiose tussen methanotrofe bacteriën en veenmossen. Allereerst is de activiteit van de methanotrofe bacteriën gemeten. De veenmossen zijn in stukken geknipt en over flessen verdeeld. Nadat de flessen waren dichtgemaakt werd methaan toegevoegd. Hierna werd bepaald welke delen van de veenmossen "actief" waren en dus waar methanotrofe bacteriën zich mogelijk ophielden.

Om de locatie en identiteit van de methanotrofe bacteriën te bepalen werd DNA (erfelijk materiaal) geïsoleerd. De 16S ribosomale RNA genen werden geamplificeerd en getransformeerd in *Escherichia coli*. Met de 16S ribosomale RNA gen sequenties werd een verwantschapsonderzoek uitgevoerd en de dominante 16S rRNA gen sequentie van een methanotrofe bacterië werd gebruikt om specifieke fluorescent gelabelde oligonucleotiden te maken. Fluorescente *in situ* hybridizatie liet zien dat deze oligonucleotiden hybridizeerden met de dominante methanotrofe bacteriën in het mos. Deze methanotrofe bacteriën leven met name op de bladeren en in de stelen van de veenmossen.

Het uiteindelijke bewijs van de symbiose werd geleverd door te laten zien dat de methanotrofe bacteriën in de veenmossen ^{13}C-verrijkt methaan omzetten in koolstofdioxide. Dit koolstofdioxide was hierdoor vervolgens ook verrijkt in ^{13}C en werd door de veenmossen ingebouwd in plantbiomassa. Op deze manier werd bewezen dat tussen de 5 en 20 procent van de koolstof die de veenmos opneemt in de natuur, aangeleverd wordt door de methanotrofe bacterie-symbionten.

Hoofdstuk drie van dit proefschrift beschrijft de isolatie van een methaanetende bacterie uit het veenmos *Sphagnum cuspidatum*. *Methylosinus acidiphilus* is een nog niet eerder beschreven methaanetende bacterie, die optimaal groeit bij een zuurgraad van pH 5.

De microbiële diversiteit van twee veengebieden, de Mariapeel (Limburg) en het Wierdenseveld (Overijsel), wordt beschreven in hoofdstuk vier. Met behulp van het 16S ribosomale RNA gen werd een overzicht gemaakt van welke bacteriën voorkomen in deze twee veengebieden. In beide veengebieden kwamen min of meer dezelfde bacteriesoorten voor. Naast de methanotrofe bacteriën kwamen de *Acidobacterium*-achtigen en de

Planctomyceten het meeste voor. De rol van deze twee groepen in het veen is tot nu toe onbekend, maar hun aanwezigheid in veengebieden over de wereld duidt erop dat ze betrokken zouden kunnen zijn bij de omzetting van C_1-koolstofverbindingen. Met behulp van electronenmicroscopie is verder gekeken naar de morfologie van de verschillende bacteriën die op of in het veenmos voorkomen.

Hoofdstuk vijf beschrijft de ontdekking van een nieuw microbiologisch proces; namelijk de oxidatie van methaan gekoppeld aan denitrificatie. Dit is een nieuw proces waarbij micro-organismen zonder zuurstof de omzetting van methaan en nitriet/nitraat katalyseren waarbij koolstofdioxide en stikstofgas worden gevormd. De ontdekking begon met het verzamelen van sediment (modder) uit het Twentekanaal waar methaan en nitraat tegelijkertijd voorkomen. Het sediment werd vervolgens meer dan een jaar lang gevoed met methaan en nitriet/nitraat in een laboratorium opstelling die volledig luchtdicht werd gehouden. Na 16 maanden kon door massabalansen de stoichiometrie van de nitriet/nitraat afhankelijke methaanomzetting worden vastgesteld. De verhouding van omgezet methaan en nitriet/nitraat klopte met de theoretische verhouding. Ook de hoeveelheid stikstofgas die werd geproduceerd was gelijk aan die van de theorie.

Evenals de studie met veenmossen kon ook hier met behulp van ^{13}C-methaan worden aangetoond dat het ^{13}C-methaan terecht kwam in de vetzuur fractie van de microbiële biomassa. De omzetting bleek te worden uitgevoerd door een tot nu toe onbekend consortium van twee soorten micro-organismen. Ongeveer 10% van de populatie bestond uit methanotrofe Archaea, ver verwant van de Archaea uit de oceaan die een rol spelen in de sulfaat-afhankelijke methaanomzetting. Verder bleek de populatie voor 80% te bestaan uit een bacterie zonder bekende familieleden.

Uit moleculair-ecologische studies gedaan aan zoetwater ecosystemen over de hele wereld, blijkt dat de nieuwe Archaea en bacterie op veel plaatsen op aarde voorkomen. Dit duidt erop dat het proces van nitriet/nitraat-afhankelijke methaanomzetting een wereldwijde bijdrage kan leveren aan het terugdringen van methaanemissie uit zoetwatersedimenten. De ontdekking van de twee nieuwe processen van microbiële methaanoxidatie toont aan dat de methaancyclus op de aarde complexer is dan tot nu toe werd gedacht. Deze kennis zou bijvoorbeeld gebruikt kunnen worden om de klimaatmodellen te verbeteren.

summary

new directions in microbial methane oxidaton

Methane is a greenhouse gas of global importance; therefore the biological methane cycle has been the focus of many studies. Methane is biologically produced by micro-organisms and as recently discovered by plants. Methane oxidation is the microbiological conversion of methane into carbon dioxide. Biogeochemical controls on the oxidation of methane are poorly understood. Further insight in this process is important for our understanding of methane cycling in natural ecosystems and for microbial diversity in general. Until now, it was assumed that microbial methane oxidation could only proceed with oxygen (aerobic) or sulphate (anaerobic).

The research in this PhD thesis aimed at discovering new micro-organisms that use methane as energy source in both oxic and oxygen-limited ecosystems. Large amounts of methane formed in peat ecosystems are recycled and do not reach the atmosphere. This phenomenon was not well understood and chapter two of this thesis describes the discovery of a major methane sink in peat ecosystems. Submerged *Sphagnum* mosses, the dominant plants in peat ecosystems, consume methane through symbiosis with partly endophytic methanotrophic bacteria, leading to highly effective *in situ* methane recycling. Based on 16S rRNA molecular probes, the identity and presence of these methanotrophic bacteria in the hyaline cells of the plant and on stem leaves, was shown.

Incubation with ^{13}C-methane showed rapid *in situ* oxidation of methane by these bacteria to carbon dioxide which was subsequently fixed by *Sphagnum* as shown by incorporation of ^{13}C-methane into plant sterols. In this way, methane acts as a significant 5-20% carbon source for *Sphagnum*. The symbiosis explains both the efficient recycling of methane and the high organic carbon burial in these wetland ecosystems.

Attempts to isolate methanotrophic bacteria in these mosses resulted in the isolation of a new acid-tolerant aerobic methane-oxidizing bacterium, *Methylosinus acidiphilus*. This bacterium grows between pH 4.5 and 6.5, with an optimum at pH 5.4 and is the first acidic member of the *Methylosinus-Methylocystis* group of methane oxidizers. Fluorescence *in situ* hybridisation (FISH) showed that the abundance of *Methylosinus acidiphilus* in *Sphagnum* mosses was 10% of the total bacterial population. *Methylosinus acidiphilus* was not the dominant methanotroph in this habitat because an uncultivated acidophilic type II methanotroph made up 60% of the total community.

Interestingly, the major fatty acids of *Methylosinus acidiphilus*, 16:1ω7 and 18:1ω7 were the same as those of other acidophilic methanotrophs, whereas other *Methylosinus* species only have low levels of these fatty acids.

Chapter four of this thesis characterizes the microbial diversity of microbes inhabiting two Dutch *Sphagnum* peat ecosystems. The first peat system, the Mariapeel, was analyzed in two successive years. Comparison of the 16S rRNA gene sequences revealed that the microbial diversity in the two years was very similar. The 16S rRNA gene libraries were dominated by representatives of the *Proteobacteria*, *Acidobacteria*, *Planctomycetes*, *Actinobacteria* and unclassified clones.

The second peat system, the Wierdense Veld, contained the same dominant phylogenetic groups as the Mariapeel. The 16S rRNA gene sequences of the three major groups, the alpha type II methanotrophs, the *Acidobacteria/Holophaga* and the *Planctomycetes* were used to design specific oligonucleotide probes for FISH. *In situ* hybridizations showed a numerical dominance of the methanotrophs, and a high morphological diversity of the *Planctomycetes* inside the *Sphagnum* mosses. Hybridization of the *Acidobacteria/Holophaga* probes gave only faint signals.

The morphological diversity of microorganisms in and on the *Sphagnum* mosses was confirmed by thin section electron microscopy. In addition, a clone library using *Planctomycetes* specific PCR primers was made from the mosses of the Mariapeel. The retrieved sequences confirmed the high abundance and diversity of *Planctomycetes* in the peat ecosystem.

Chapter five, the final chapter of this thesis, describes the discovery of a new microbial process which constitutes a new sink for methane. Modern agriculture has accelerated biological methane and nitrogen cycling on a global scale. Freshwater sediments often receive increased downward fluxes of nitrate from agricultural run-off and upward fluxes of methane generated by anaerobic decomposition. In theory, prokaryotes should be capable of using nitrate to oxidize methane anaerobically, but such organisms have neither been observed in nature nor isolated in the laboratory. Microbial oxidation of methane was thus believed to proceed only with oxygen or sulphate.

In this chapter it was shown that the direct, anaerobic oxidation of methane coupled to denitrification of nitrate is possible. A microbial consortium, enriched from anoxic sediments of the 'Twentekanaal', oxidized methane to carbon dioxide coupled to denitrification in the complete absence of oxygen. The consortium consisted of two microorganisms, a bacterium representing a phylum without any cultured species and an archaeon distantly related to marine methanotrophic Archaea. The detection of relatives of these prokaryotes in different freshwater ecosystems worldwide indicates that the here presented reaction may make a substantial contribution to the biological methane and nitrogen cycles. It shows that the microbial methane cycle is more complex than previously assumed. This complexity needs to be addressed, and the knowledge can for example be used to improve mathematical models that predict global warming in the future.

chapter 1

introduction

outline

This thesis describes the discovery of two unexpected new microbial sinks for methane. The research was performed from 2001 to 2005 at the Department of Ecological Microbiology, Institute for Water and Wetland Research, Radboud University Nijmegen.

The discovery of these two new methane sinks has provided new insight in the microbial methane cycle. Chapter one describes the general microbiology of the methane cycle. Chapter two describes the first methane sink, a new symbiosis between peat mosses and methane consuming bacteria (Raghoebarsing *et al*., 2005). Chapter three describes the isolation of a new acidiphilic methane consuming bacterium from these peat mosses (Raghoebarsing *et al*., submitted). In chapter four, the molecular microbial diversity in two Dutch peat bogs is presented (Raghoebarsing *et al*., submitted). The final chapter describes the second new methane sink. This chapter provides experimental evidence for a completely new microbial process, the nitrate dependent anaerobic oxidation of methane, which may play an important role in the global carbon cycle (Raghoebarsing *et al*., 2006).

biological methane cycle

Methane (CH_4) is after carbon dioxide (CO_2) and water vapor, the most abundant trace gas in the atmosphere (Wuebbles & Hayhoe, 2002). Methane has a strong infrared absorption and directly affects the climate as a greenhouse gas (Frankenberg *et al*., 2005). As a greenhouse gas, CH_4 is 20 times more effective than CO_2. It is therefore important to understand biological sources and sinks for methane.

In the methane cycle (Figure 1), methane is produced anaerobically by methanogenic Archaea (Schink, 1997; Thauer, 1998) and as recently discovered, aerobically by plants (Keppler *et al*., 2006). Methane is consumed both aerobically and anaerobically by microorganisms. Aerobically, methane is oxidized by methanotrophic *alpha*- and *gammaproteobacteria* (Hanson & Hanson, 1996) and anaerobically, by a consortium of yet uncultivated methanotrophic Archaea and sulphate-reducing bacteria (Orphan *et al*., 2002; Valentine, 2000; Boetius *et al*., 2000).

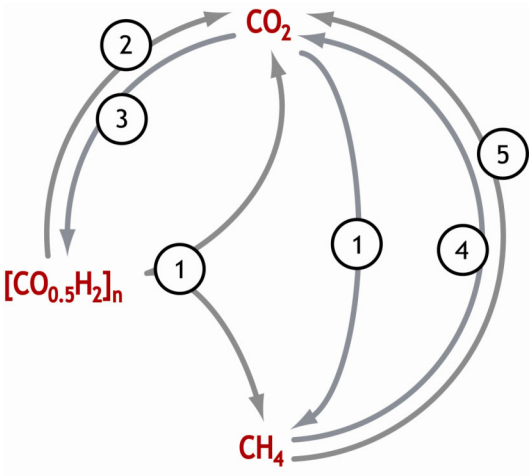

Figure 1: Methane cycle.

1. Methanogenesis; 2. Aerobic methanotrophy; 3. CO_2 fixation;
4. Aerobic chemoorganoheterotrophy; 5. Anaerobic methane oxidation

anaerobic methane production by methanogenic Archaea

Anaerobically, methane is produced by a group of Archaea, the methanogens. Methanogenic Archaea represent a large and diverse group of strictly anaerobic microorganisms which produce methane as the end product of their metabolism.

Phylogenetic analysis on the 16S rRNA gene level revealed five different orders of methanogens, *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanopyrales* (Figure 2).

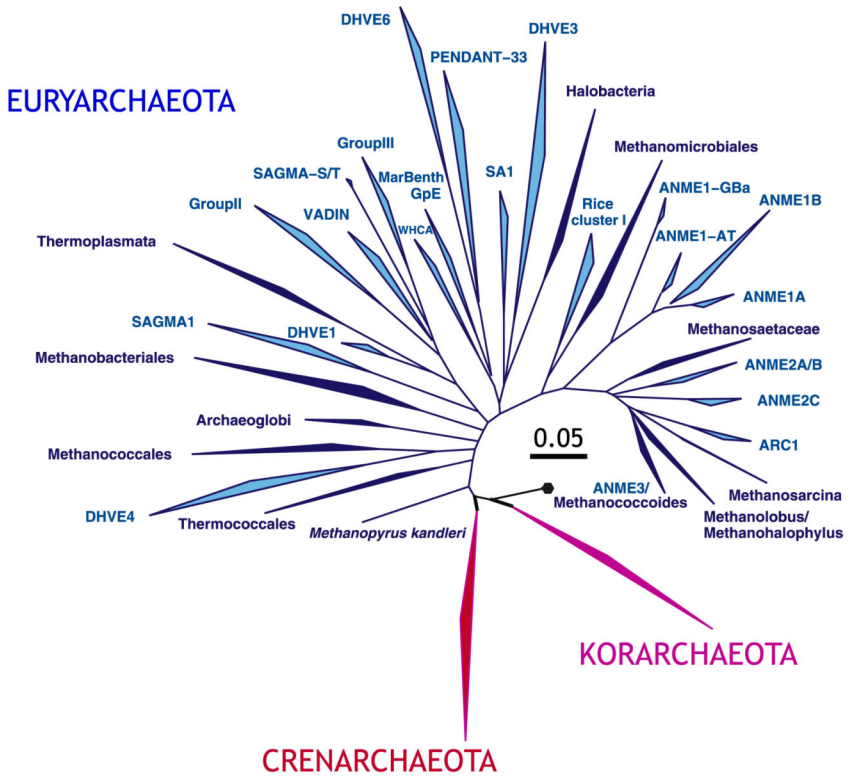

Figure 2: Domain Archaea
A 16S rRNA tree of the Archaea with the methanogenic Archaea and the methanotrophic Archaea (Schleper *et al.*, 2005)

Methanogens use at least 11 substrates, which can be clustered into three groups: CO_2-type substrates, methyl substrates and acetoclastic substrates (Brock, 2006; Segers, 1998). With a CO_2-type substrate, the methanogens are autotrophic, with CO_2 serving as both carbon source and electron-acceptor and hydrogen (H_2) serving as the energy source (electron donor), (eq.1).

Methanogenesis: $CO_2 + 4H_2 = CH_4 + 2H_2O$ (eq. 1)

All known methane producing Archaea express the enzyme methyl-coenzyme M reductase (MCR). The operon that encode MCR consist of two alpha (*mcrA*), a beta (*mcrB*) and a gamma (*mcrG*) subunits and is used as a functional gene marker for methanogenic Archaea (Hallam *et al.*, 2003; Ferry, 1999; Thauer, 1998). MCR catalyses the final step of methanogenesis, the reduction of methyl-coenzyme M ($CoM\text{-}S\text{-}CH_3$) with coenzyme B (H-S-CoB), yielding CH_4 and the heterodisulphide (CoM-S-S-CoB) (Thauer, 1998). MCR is a 300 kDa enzyme composed of three different subunits ($\alpha_2\beta_2\gamma_2$) and two tightly but non-covalently bound molecules of a nickel porphinoid, cofactor F_{430}, with a molecular mass of 905 Da (Krüger *et al.*, 2003).

Methanogenesis is relatively more important in freshwater and terrestrial environments than in oceans. This is because marine waters and sediments contain high levels of sulphate and sulphate-reducing bacteria compete with the methanogenic populations for acetate and H_2 (Kristjansson *et al.*, 1982). In freshwater systems, the substrates hydrogen and acetate are most abundant and formed by fermentation of hydrolysed organic matter. The microbial production of methane is controlled by the absence of oxygen and the amount of easily degradable organic matter (Segers, 1998). The release of methane to the atmosphere has increased through human activity. The largest anthropogenetic sources are fossil fuel production, ruminants, rice cultivation, biomass burning and waste handling (Frankenberg *et al.*, 2005; Bastviken *et al.*, 2004).

aerobic methane production by plants

Recently, it was discovered that plants produce methane in small amounts aerobically (Keppler *et al.*, 2006). This was the first report on aerobic production of methane. Methane formation was observed for different plant species and detached leaf material. The release rates for plants are in the order of 12-370 ng per g (dry weight) h^{-1}. Keppler *et al.* (2006) calculated that CH_4 release by living vegetation, mostly from tropical forest and grasslands, is around 149 Tg $year^{-1}$. The estimated emissions via this new process are 10-30% of the present annual methane sources. The process of methane production by plants is yet unknown, but a plant related compound, pectin, could play an important role.

aerobic methane consumption by methanotrophic bacteria

Aerobic methanotrophic bacteria, or methane oxidizing bacteria, are unique in their ability to utilize methane as carbon and energy source with carbon dioxide (CO_2) and cell carbon as the end products (Hanson & Hanson, 1996). The methanotrophs occur in three monophyletic lineages (Figure 3) consisting of thirteen genera, belonging to the gamma

and alpha groups of the *Proteobacteria* (Dedysh *et al*., 2004; Hanson & Hanson, 1996). The first observation on aerobic methanotrophs was made already 100 years ago (Soehngen, 1906). The most recent addition to the methanotrophs was *Crenothrix polyspora*, a conspicuous filamentous bacterium with a complex life cycle (Stoecker *et al*., 2006). This hitherto uncultured bacterium occurs frequently in drinking water systems, but its phylogeny and physiology remained unresolved. Using fluorescent *in situ* hybridization and microradioautography it was shown that *C. polyspora* was a *gammaproteobacterium* closely related to methanotrophs and capable of oxidizing methane. *C. polyspora* encodes a phylogenetically very unusual particulate methane monooxygenase whose expression is strongly increased in the presence of methane (Stoecker *et al*., 2006).

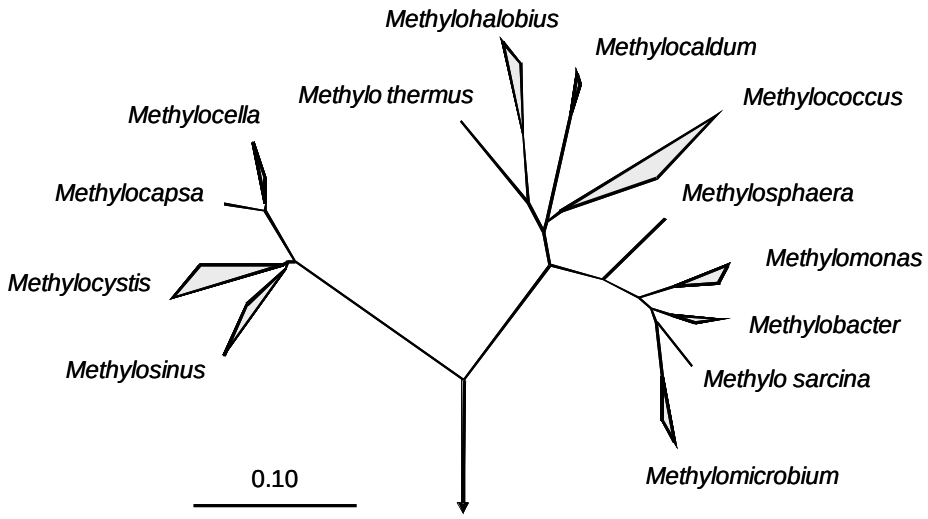

Figure 3: Methanotrophic Bacteria

The methanotrophic lineages differ in their cell morphology, DNA G+C content, the major phospolipid fatty acids, the arrangement of intracytoplasmic membranes (ICM) and the pathway for formaldehyde assimilation. The first lineage comprises type-I and type-X methanotrophs, which use the ribulose monophosphate (RuMP) pathway for formaldehyde fixation (*gammaproteobacteria*) and their ICM are arranged as bundles of disc-shaped vesicles distributed throughout the cell. The second and third lineages comprise type-II methanotrophs, which use the serine pathway for formaldehyde fixation *(alphaproteobacteria*) and their ICM's are arranged as paired membranes running along the periphery of the cell. In the serine pathway, a two carbon unit, acetyl-CoA, is synthesized, one molecule from formaldehyde and one from CO_2. The pathway requires the introduction of reducing power and energy in the form of two molecules of both NADH and ATP for each

acetyl-CoA synthesized. The RuMP is more efficient than the serine pathway in that all of the carbon atoms for cell material are derived from formaldehyde, and since formaldehyde is at the same oxidation level as cell material, no reducing power is needed. The latter two lineages include the *Methylocystis-Methylosinus* genera and the acidophilic methanotrophs, consisting of the genera, *Methylocella* and *Methylocapsa*.

The methane-oxidizing bacteria require oxygen to use methane (eq.2) and posses a specific enzyme system, methane monooxygenase (MMO). MMO catalyzes the conversion of methane into methanol (CH_3OH) by using two reducing equivalents to split the O-O bonds of dioxygen. One of the oxygen atoms is reduced to form H_2O, and the other is incorporated into methane to form CH_3OH.

Methane oxidation: $CH_4 + 2O_2 = CO_2 + 2H_2O$ (eq. 2)

Two forms of MMOs have been found in methanotrophic bacteria (Hanson & Hanson, 1996). The cytoplasmic soluble MMO (sMMO) which is NADH dependent and the membrane bound particulate MMO (pMMO) which is cytochrome c dependent. These two enzymes show no genetic or structural homology despite their similar function in the cell. The pMMO seems to be present in most methanotrophs, except in the acidophilic *Methylocella* species (Dedysh *et al*., 2004; Dunfield *et al*., 2003; Dedysh *et al*., 2000). The overall pathway of methane oxidation consists of four, two-electron oxidation steps (eq.3).

$$\underset{\text{methane}}{CH_4} \rightarrow \underset{\text{methanol}}{CH_3OH} \rightarrow \underset{\text{formaldehyde}}{HCHO} \rightarrow \underset{\text{formate}}{HCOOH} \rightarrow \underset{\text{bicarbonate/carbon dioxide}}{HCO_3^-/CO_2} \quad \text{(eq. 3)}$$

anaerobic methane oxidation

Anaerobically, methane is oxidized by a consortia consisting of yet uncultivated methanotrophic Archaea (ANME) and sulphate-reducing bacteria (SRB) (Orphan *et al*., 2002; Valentine, 2000; Boetius *et al*., 2000; Hinrich *et al*., 1999). This process is a major biological sink of methane in marine sediments (Knittel *et al*., 2005). The anaerobic oxidation of methane (AOM) involves the reversal of methanogenesis coupled to the reduction of sulphate to sulphide (Hallam *et al*., 2004). The methanotrophic Archaea appear to be organized in clusters, surrounded by their sulphate-reducing bacterial partner, but single cells have also been observed (Orphan *et al*., 2002). The possibility that one organism could catalyse the whole process has not yet been ruled out.

The methanotrophic Archaea consist of three ANME groups based on 16S rRNA gene analysis (Figure 2). The ANME-1 archaea, subgroups ANME 1a -1b, are distantly related to the orders *Methanosarcinales* and *Methanomicrobiales* (Hinrich *et al*., 1999). The other two ANME groups are ANME-2, subgroups ANME 2a-2b-2c, belonging to the order of *Methanosarcinales* (Knittel *et al*., 2005; Boetius *et al*., 2000) and ANME-3 distantly related to *Methanococcoides* spp. (Knittel *et al*., 2005). The sulphate reducing bacteria involved are members of the *Desulfosarcina-Desulfococcus* group belonging to the *deltaproteobacteria*. The pathway of sulphate dependent anaerobic methane oxidation involves the oxidation of methane to CO_2 by the methanotrophic Archaea. These methanotrophic Archaea reverse steps of methanogenesis and a reduced end product becomes the energy substrate for the sulphate reducing bacteria (eq. 4-6). The nature of the intermediate is currently debated. Some possibilities are hydrogen, formate, acetate and methylated compounds (Sørensen *et al*., 2001) or perhaps even nanowires (Ishii *et al*., 2005).

$CH_4 + 2H_2O = CO_2 + 4H_2$ (reverse methanogenesis) (eq. 4)

$4H_2 + SO_4^{2-} + H^+ = HS^- + 4H_2O$ (sulphate-reducers) (eq. 5)

$CH_4 + SO_4^{2-} + H^+ = CO_2 + HS^- + 2H_2O$ (overall) (eq. 6)

The consortia of microorganisms, members of the ANME- group and their sulphate reducing partners, capable of AOM are very difficult to cultivate because of the slow growth rate (Girguis *et al*., 2003; Michaelis *et al*., 2002). Girguis *et al*. (2005) applied high pressure reactors to cultivate these microorganisms for 29 weeks, and was able to reach about 10^7 rRNA copy numbers per ml.

Environmental genomics showed the occurrence of a new methyl coenzyme M reductase (MCR) in ANME-1 and ANME-2 archaea (Hallam *et al*., 2003). This enzyme is supposed to catalyse the final step in methanogenesis. Phylogenetic analysis of isolated novel ANME-1 and ANME-2 *mcrA* genes sequences showed that these sequences are associated with ANME-1 or ANME-2 group members (Hallam *et al*., 2003). A candidate for the nickel cofactor F_{430} of methyl coenzyme M reductase, Ni-protein I, has been isolated from Black Sea ANME communities (Krüger *et al*., 2003) and supports the involvement of MCR in AOM. The mass of the new F_{430} cofactor was 951 Da. Other genes associated with methanogenesis have been identified in the ANME-1 and ANME-2 group and therefore support the 'reverse methanogenesis' pathway for anaerobic methane oxidation (Meyerdierks *et al*., 2005; Hallam *et al*., 2004). The results of environmental genomics so provide a basis for

identifying methanotrophic archaea and define a functional genomic link between methanogenic and methanotrophic archaea (Meyerdierks *et al*., 2005; Hallam *et al*., 2004; Krüger *et al*., 2003).

scope

So far, different aspects of the methane cycle have been described. The experimental focus of this thesis has been on aerobic and anaerobic methane sinks in Dutch wetlands. Microorganisms remove methane from the environment through aerobic methane oxidation with oxygen (Hanson & Hanson, 1996) and anaerobic methane oxidation with sulphate (Orphan *et al*., 2002; Boetius *et al*., 2000). At present, the emissions of methane to the atmosphere have almost doubled due to anthropogenic activities (Frankenberg *et al*., 2005). Wetlands, which are the focus of this study, contribute between 15 to 45% to the global methane emissions (Segers, 1998). The balance between methane production and methane consumption determines whether a particular environment acts as a source or a sink for atmospheric methane (Bodelier *et al*., 2005). The research described in this thesis aimed to identify new CH_4 sinks and the microorganisms involved in these processes.

In wetlands, such as peat bogs, the methane cycle has been studied with respect to global climate change (Smith *et al*., 2004; Dedysh *et al*., 1998). Peat bogs are both a source and a sink for the greenhouse gases CO_2 and CH_4 (Yavitt *et al*., 1997; Bridgeham & Richardson, 1992; Gorham, 1991). Peat bogs are ombrotrophic (only fed by rainwater and dry atmospheric decomposition), acidic and dominated by peat mosses from the genera *Sphagnum*. In these systems, CH_4 produced by methanogenic Archaea and CO_2 are the end-products of anaerobic degradation of organic matter. The CH_4 produced is consumed by methanotrophic bacteria resulting in CO_2. In this way, methane is removed effectively in these ecosystems by microorganisms and as already mentioned peat bogs can even be a sink for atmospheric methane. The Mariapeel, a *Sphagnum* bog remnant in the Netherlands, was used as an example to study this process.

Many reports describe the process of sulphate dependent AOM in marine environments (Strous & Jetten, 2004; Boetius *et al*., 2000; Hinrich *et al*., 1999). In these systems, oxygen is depleted and CH_4, which is produced in deep sediments, diffuses upwards. Before the methane reaches the water column it is consumed by sulphate dependent AOM. This process is carried out by symbioses of two prokaryotes. The Gibbs energy change (ΔG°) is calculated to be around -20 to -40 kJ/mol, at *in situ* conditions. There are a number of reports describing sulphate dependent AOM in non-marine environments, such as landfills (Grossman *et al*., 2002) and terrestrial mud volcanoes (Alain *et al*., 2005). Recently, the presence and activity of sulphate dependent AOM was also found in a freshwater lake (Eller *et al*., 2005), suggesting a widespread distribution.

In Dutch canals large amounts of organic matter accumulate from high primary production. Both CH_4 and CO_2 are the end-product of the anaerobic degradation of the organic matter. These canals also contain high levels of nitrate (0.5-2 mM) which originate from agricultural runoff. In this ecosystem CH_4 and nitrate coexist, and microorganisms could oxidize CH_4 using nitrate as electron acceptor.

Theoretically, methane can be used as a carbon source for denitrification (Zehnder & Brock, 1980). The Gibbs energy change with nitrate as an oxidant is almost equivalent to the change in free energy with oxygen. The energy yield for sulphate is much less favourable than for oxygen and nitrate (Zehnder & Brock, 1980). An ecosystem were CH_4 and nitrate coexist could therefore be a possible sink for methane. Sediment from the Twentekanaal a Dutch canal was used to study the process of nitrate dependent AOM as described in this thesis.

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chapter 2

methanotrophic symbionts provide carbon for photosynthesis in peat bogs

abstract

Wetlands are the largest natural source for atmospheric methane (Hein *et al*., 1997), the second most important greenhouse gas (Rodhe, 1990), and its fluxes to the atmosphere depend strongly on the climate (Smith *et al*., 2004). However, by far the largest part of the methane formed in these ecosystems is recycled and does not reach the atmosphere (Dedysh *et al*., 1998; Lamers *et al*., 1999). The biogeochemical controls on the efficient oxidation of methane are still poorly understood. Here we show that submerged *Sphagnum* mosses, the dominant plants in this habitat, consume methane through symbiosis with partly endophytic methanotrophic bacteria, leading to highly effective *in situ* methane recycling. Molecular probes revealed their presence in the hyaline cells of the plant and on stem leaves. Incubation with ^{13}C-methane showed rapid *in situ* oxidation by these bacteria to carbon dioxide which was subsequently fixed by *Sphagnum* as shown by incorporation of ^{13}C-methane into plant sterols. In this way, methane acts as a significant (10-15%) carbon source for *Sphagnum*. The symbiosis explains both the efficient recycling of methane and the high organic carbon burial in these wetland ecosystems.

methods

in situ conditions

In the Mariapeel nature reserve (the Netherlands; 51°24'90''N; 5°54'90''E) δ^{13}C values of *Sphagnum* mosses and material from the decaying peat were determined on freeze-dried homogenized material according to Marguillier *et al*. (1997). Concentrations and δ^{13}C values of dissolved carbon dioxide and methane were measured as decribed previously (Smolders *et al*., 2002).

methane oxidation

Potential methane oxidation of different parts of *Sphagnum* were measured by incubating 6 g of thoroughly washed *Sphagnum* in 100 ml infusion flasks sealed with airtight rubber stoppers. To prevent mass transport limitations, no additional water was added to the experiments. To each flask 1 ml of pure methane was added and methane consumption was measured every 6 h during two days. Methane oxidation rates were calculated by linear regression. Ten-fold concentrated water samples (10^6 bacterial cells/ml) from the bog were used as controls and showed no methane oxidation. Samples were collected in the Netherlands from 7 lawn locations (*S. magellanicum , S. papillosum*) and 6 bog pools (*S. cuspidatum*), one of these being the Mariapeel. Methane was measured on a HP 5890 gas chromatograph equipped with a flame ionization detector and a Porapak Q column (80/100 mesh).

16S rRNA gene sequence analysis, FISH and electron microscopy

Total genomic DNA from *S. cuspidatum* plants containing methanotrophs, isolated with combined methods (Lomans *et al*., 2001), was used as template for PCR amplification of 16S rRNA genes. PCR was performed with general bacterial primers (Juretschko *et al*., 1998) using a T gradient thermal cycler (Biometra, Germany) and, subsequently a clone library was made as described previously (Juretschko *et al*., 1998). Based on the obtained 16S rRNA gene sequences two new oligonucleotide probes S-*-18ALF-0218-a-A-18 (5'-GGGCCGATCCCCCGGCGA-3') and S-*-18ALF-1437-a-A-18 (5'-CTTGCGGTTAACAGAACG-3') were designed using the ARB program package (Ludwig *et al*., 2004). Besides these species-specific probes we used group-specific probes described in literature (Amann *et al*., 1990; Daims *et al*., 1999). Fresh *S. cuspidatum* stems sectioned with a scalpel (section thickness 0.1 ± 0.05 mm) were used for FISH as described previously (Amann *et al*., 1990). Formamide concentrations used in the FISH experiments varied between 10 and 20%. No signal was obtained at these formamide concentrations when testing the specificity of the probes with *Beyerinckia indica* subsp. *indica* (DSM 1715), which has the fewest mismatches of all reference organisms to the designed probes. Electron microscopy (TEM/SEM) was performed on stems and stem leaves following published protocols (Dedysh *et al*., 2000; Wolters-Arts *et al*., 2002).

methane incorporation measurements

S. cuspidatum, collected from the Mariapeel nature reserve, was washed with demineralized water and incubated in 250-ml serum bottles in 5 g wet weight aliquots with 150 ml medium described previously (Dedysh *et al*., 2000). ^{13}C- or ^{12}C-CH_4 or CO_2 were added to final concentrations of 200 µM as specified in the text. The bottles were shaken at 150 rpm at ambient conditions and sacrificed for lipid analysis at days 0, 1, 3 and 5. Lipids were ultrasonically extracted and analysed by gas chromatography/mass spectrometry and isotope ratio gas chromatography mass spectrometry as described by Schouten *et al*. (1998). Hopanes were analysed by treatment of the lipid fraction with periodic acid and sodium borohydride as described previously (Rohmer *et al*., 1992; Sinninghe Damsté *et al*., 2004).

isotopic mass balancing

The measured $\delta^{13}C$ values of *S. cuspidatum* in the field (-26 ‰, Table 1) resulted from assimilation of dissolved carbon dioxide (-14.5 ‰), respired methane (-56 ‰) and fractionation against ^{13}C during (mass transfer limited) carbon dioxide fixation (7 ± 3 ‰) (Keeley & Sandquist, 1992; Smolders *et al*., 2003). The following equation describes this

relationship quantitatively (where *a* denotes the fraction of plant carbon derived from methane and *Ep* denotes the fractionation during fixation):

$$\delta^{13}C(Sphagnum) = a\delta^{13}C(\text{respired methane}) + (1 - a)\delta^{13}C(\text{carbon dioxide}) - Ep \quad \text{(eq.1)}$$

Because all $\delta^{13}C$ values from equation 4 were known experimentally, it could be derived that the contribution of methane to *Sphagnum* carbon (a) was between 0.05 and 0.2 (equivalent to 5-20%).

results & discussion

Peat bogs alternate between lawns and pools; lawns are dominated by species that grow up to several decimetres above the water table. Pools are dominated by aquatic species such as *Sphagnum cuspidatum*, which form layers of living plants below the water table. We investigated the methane oxidizing activity of submerged *S. cuspidatum* from bog pools at different field locations in the Netherlands and compared it to the activity of *S. magellanicum* and *S. papillosum* growing in lawns. The potential methane oxidizing activity was substantially higher in the submerged mosses (Figure 1). In control experiments with bog water without *Sphagnum*, methane was not oxidized, indicating that the methanotrophic bacteria were mainly present on or in the living *Sphagnum* tissue.

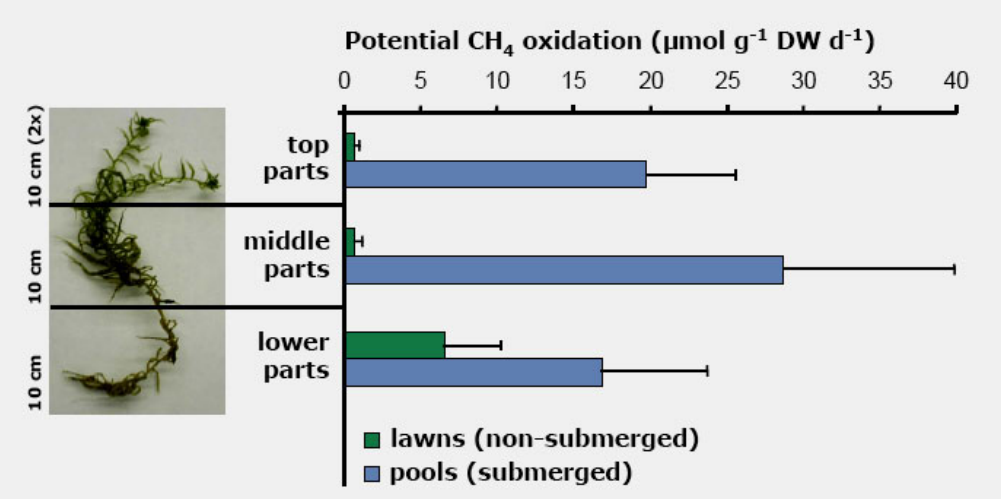

Figure 1: Methane oxidation potential of different parts of submerged and non-submerged *Sphagnum* mosses as a measure of methanotrophs associated. Error bars indicate standard deviations of at least six independent experiments.

The identity and location of these methanotrophs was determined in a molecular approach. Total genomic DNA from washed *Sphagnum* plants was isolated and bacterial 16S ribosomal RNA genes were amplified, cloned into *E. coli*, sequenced and analyzed phylogenetically. One of the 16S rRNA gene sequences of the clone library was affiliated to a cluster of type II methanotrophs that contained acidophilic methanotrophs isolated from *Sphagnum* bogs, such as *Methylocella palustris* (identity 93%) (Dedysh *et al*., 2000), and *Methylocapsa acidophila* (identity 93%) (Dedysh *et al*., 2002).

The full 16S rRNA gene sequence was used to design two specific oligonucleotide probes for fluorescence *in situ* hybridization (FISH). FISH was combined with serial sectioning of the stems and the stem leaves of multiple individuals of submerged *S. cuspidatum.* The methanotroph targeted by the probes was the dominant methanotroph in *S. cuspidatum* sections, accounting for over 75% of all *alphaproteobacteria*. Application of general probes showed that the *alphaproteobacteria* themselves made up 80% of all detected bacteria, indicating that the new methanotroph was indeed the dominant bacterium in *S. cuspidatum* sections. *Gammaproteobacteria* (including type I methanotrophs) were virtually absent.

In *S. cuspidatum* stems, clusters of the new methanotroph were present in the hyaline cells of the outer cortex (Figure 2a-c, in total 10^6 - 10^7 methanotrophs per individual plant, total length of stem ~40 cm). Hyaline cells are dead cells, filled with water, which contain pores by which solutes (and bacteria) can move in or out (Rydin & Clymo, 1989). The presence of clusters indicated that this bacterium was actively growing inside the hyaline cells. The bacterial clusters consisted of 5-25 individual coccoid cells lying closely together in a random arrangement. On the stem leaves, the same probes hybridised with bacteria occurring as dense, geometric clusters tightly bound to the living plant cells (Figure 2d-e, 10^5 - 10^6 methanotrophs per individual plant).Differences in the morphology of micro-colonies have been observed previously to depend on environmental conditions for other microorganisms (Yao *et al*., 1992). On the basis of the measured *in vitro* methane oxidizing capacity of *S. cuspidatum* (~ 20 µmol/g dry weight/day; Figure 1) and the number of methanotrophs per plant, an activity in the order of 1- 4 fmol methane/cell/h was estimated for the associated methanotrophs. This is significantly higher than the *in vitro* methane oxidation rates reported for acidophilic methanotrophs (~0.3 fmol methane/cell/h), (Dedysh *et al*., 2000), indicating that the actual numbers of methanotrophs per *S. cuspidatum* individual might still be underestimated.

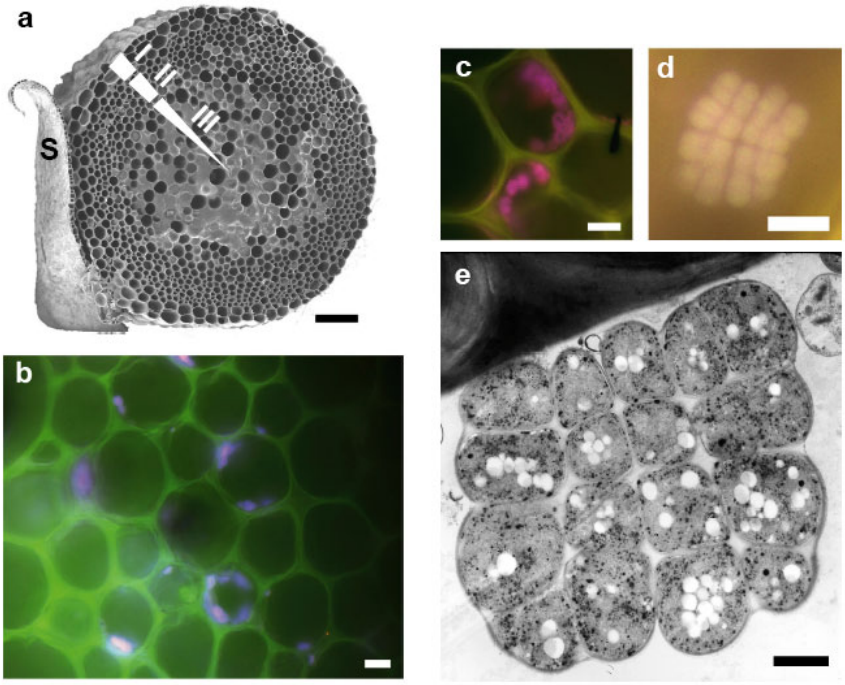

Figure 2: *In situ* detection of the new methanotroph in *S. cuspidatum* with fluorescently labelled rRNA-targeted oligonucleotide probes. (a) Cryo-scanning electron micrograph of a stem cross section. S, stem leaf; I, outer cortex; II, internal cylinder; III, inner pith. Scale bar 100 µm. (b), (c) Epifluorescence micrographs of the new methanotroph (purple or pink cells) in the outer cortex of *Sphagnum* stems, after a double hybridization with the specific probe 18ALF1437 and the general probe EUB. Scale bars 10 and 5 µm. (d) Dense, geometric clusters of the same bacterium on a stem leaf, after a triple hybridization with the specific probe18ALF1437, the general probe EUB and probe ALF968 (specific for *alphaproteobacteria*). Scale bar 5 µm. (e) Transmission electron micrograph of a geometric cluster closely attached to a stem leaf. Scale bar 1 µm. (see page 102 for color figure).

Because FISH had shown that the new methanotroph was the only bacterium occurring in the characteristic geometric clusters, it was possible to unambiguously identify and inspect this bacterium with transmission electron microscopy (TEM). The TEM and FISH results were consistent with respect to the localization of the new methanotroph. TEM also showed that this bacterium did not contain any intracytoplasmic membranes. The absence of intracytoplasmic membranes was noted previously for the phylogenetically related type II methanotroph M. palustris (Dedysh *et al*., 2000). Otherwise, intracytoplasmic membranes are a characteristic feature of methanotrophic bacteria.

The predominance of type II methanotrophs was further substantiated by the presence of bishomohopanoic acid in *Sphagnum* lipid extracts after periodic acid treatment. This compound was previously shown to be formed after periodic acid treatment from the C_{35} hopanetetrol derivatives, membrane rigidifiers produced by methanotropic bacteria

(Rohmer *et al*., 1992). The natural ^{13}C-contents of this compound ($\delta^{13}C$ = -39.8 ‰) was substantially depleted relative to *Sphagnum* cell material and enriched compared to that of methane (Table 1), in accordance with its origin from serine-cycle (type II) methanotropic bacteria (Jahnke *et al*., 1999). Using this methanotrophic biomarker it was determined whether the methanotrophs associated with *Sphagnum* were actively growing. After incubating *Sphagnum* with ^{13}C-labelled methane for five days, isotopic analysis showed that ^{13}C-labelled methane was incorporated into this lipid in substantial amounts; nearly 50% of this lipid was biosynthesized from the labelled methane, indicating that the methanotrophic population had doubled over the course of the experiment.

Table 1 Methane and CO_2 concentrations and $\delta^{13}C$ values in the Mariapeel bog pool.

	CH_4	CO_2	plants*
sediment gas composition (%)	52	48	-
bulk water concentration (µM)	50±20	160±30	-
$\delta^{13}C$ (‰)	-56	-14.5	-26.5

* The $\delta^{13}C$ of growing (-26 ‰) and decaying (-27 ‰) *S. cuspidatum* were almost identical.

The observed tight association of methanotrophs with *S. cuspidatum* would enable the efficient recycling into living mosses of both oxygen (derived from photosynthesis) and methane (derived from decaying plants), according to the following set of equations:

CH_4 oxidation:	$CH_4 + 2\ O_2$	$= CO_2 + 2\ H_2O$	(eq.2)
CO_2 fixation:	$2\ CO_2 + 2\ H_2O$	$= 2\ CH_2O + 2\ O_2$	(eq.3)
balance:	$CH_4 + CO_2$	$= 2\ CH_2O$	(eq.4)

To provide experimental evidence for this scenario, the potential contribution of methane to carbon fixation by *S. cuspidatum* was investigated under conditions relevant to the field. Multiple batches of individuals of *S. cuspidatum* were incubated with ^{13}C labelled methane in the presence of unlabeled carbon dioxide. As a control experiment, only ^{13}C labelled carbon dioxide was supplied. Both compounds were added to a final concentration of 0.2 mM, close to the *in situ* concentrations (Table 1). Over 5 days, incorporation of the label by *S. cuspidatum* was determined via the ^{13}C incorporation into sitosterol, a *Sphagnum*-specific sterol (Figure 3). Methane was assimilated into the sitosterol pool at a rate of 0.20 ± 0.03 µg C/g dry weight/day, compared to 1.4 ± 0.1 µg C/g dry weight/day for carbon dioxide. Thus, in the presence of carbon dioxide, at near situ concentrations, the capacity of methane incorporation by *S. cuspidatum* was ~15% of the carbon dioxide assimilation capacity.

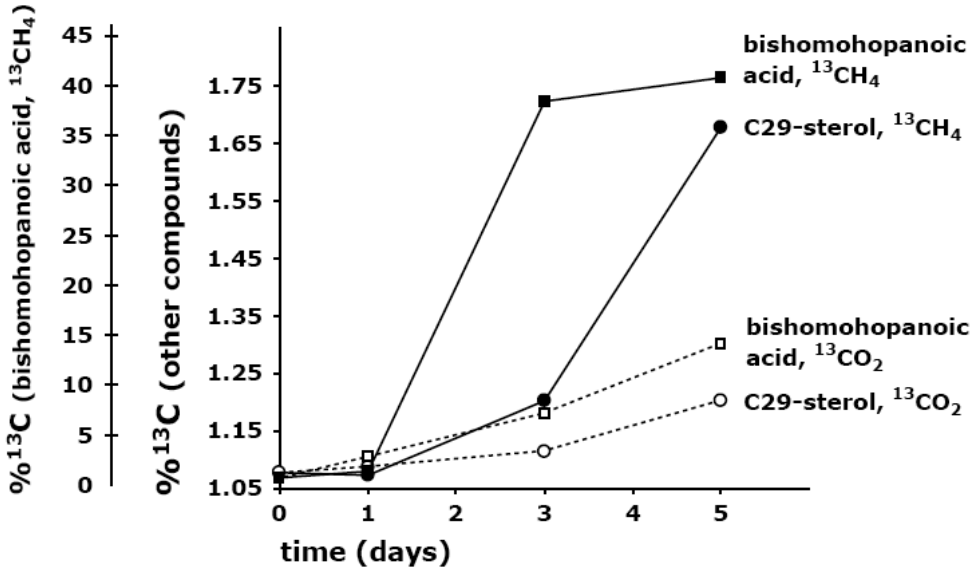

Figure 3: Incorporation of ^{13}C label in biomarkers for *Sphagnum* (circles) and methanotrophic bacteria (squares). Filled circles/solid lines show the results for labeled methane (99 % ^{13}C) in the presence of unlabeled carbon dioxide; open symbols/dashed lines show the results for labeled carbon dioxide (4 % ^{13}C).

The natural carbon isotope abundances of *Sphagnum* mosses in the field ($\delta^{13}C$ -26.5 ‰; Table 1) are consistent with our estimate that 15% of the carbon fixed by *Sphagnum* derives from isotopically depleted methane (i.e. -56‰; Table 1). *S. cuspidatum* fixes carbon dioxide via the Calvin cycle and is able to fractionate strongly against ^{13}C (up to 29 ‰) at high carbon dioxide concentrations (> 2 mM), (Keeley & Sandquist, 1992; Smolders *et al*., 2003). However, unlike vascular (semi-) aquatic plants such as rice, *S. cuspidatum* does not have aerenchyma (Rydin & Clymo, 1989) that facilitate the transport of atmospheric carbon dioxide. Therefore, at lower carbon dioxide concentrations, carbon assimilation by *S. cuspidatum* is limited by mass transfer and carbon fractionation has been reported to decrease to at most 4 ‰ (Keeley & Sandquist, 1992; Smolders *et al*., 2003). Because the average carbon dioxide concentration in the field was approximately 0.16 mM, a range of 4-10 ‰ was used as a conservative estimate for carbon fractionation by *S. cuspidatum* in the field (Keeley & Sandquist, 1992; Smolders *et al*., 2003). With this assumption, the data from Table 1 and a simple isotopic mass balance (Methods), we calculated that methane contributed on average between 5 and 20% to the carbon fixed by *S. cuspidatum* in the field, in good agreement with the labeling results. It is likely that variation in local conditions (water depth, exposure to wind, temperature, light availability, rates of methane ebullition compared to diffusion/advection) will effect the

relative contribution of methane to the carbon uptake of *Sphagnum* mosses in space and time. This will also be determined by the location of the symbiotic methanotrophs in the plants, both in the direct vicinity of the photosynthetically active cells and in the more remote hyaline cells of the stems.

Our results show that methane is a significant and as yet overlooked supplement to the carbon intake of submerged *S. cuspidatum* in peat bogs. Peat bogs in the Northern hemisphere store up to one third of the carbon sequestered in soils globally (Post *et al*., 1982). This is surprising considering that the primary production is limited by the nutrient delivery through rain water and the limited delivery of carbon dioxide to the acidic waters of these ecosystems (Lamers *et al*., 1999). The efficient recycling of peat decomposition products (such as methane) as demonstrated here may mechanistically explain the paradox of peatlands as ecosystems with apparent low primary productivity combined with high carbon burial.

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chapter 3

Methylosinus acidiphilus sp. nov.,

a new methane-oxidizing acid-tolerant bacterium

isolated from a *Sphagnum* peat bog

abstract

A new acid-tolerant aerobic methane-oxidizing bacterium, strain 29, was isolated from an acidic *Sphagnum* peat bog, at Mariapeel, the Netherlands. Strain 29 grows between pH 4.5 and 6.5, with an optimum at pH 5.4. Surprisingly, the 16S rRNA, *pmoA* and *mmoX* gene sequences were affiliated to the type-II methanotrophs of the *Methylosinus-Methylocystis* group within the *alfaproteobacteria* and not to the known subcluster of acidophilic methanotrophs such as *Methylocella* and *Methylocapsa*. On basis of the 16S rRNA gene, strain 29 was most closely related to *Methylosinus sporium*, with a similarity of 98.6%. Consistently, the intracytoplasmic membrane system (ICM) of strain 29 was very similar to that of type-II methanotrophs. Fluorescence *in situ* hybridisation (FISH) showed that the abundance of strain 29 in *Sphagnum* mosses was 10% of the total bacterial population. Strain 29 was not the dominant methanotroph in this habitat because an uncultivated acidophilic methanotroph made up 60% of the total community. Interestingly, the major fatty acids of strain 29, 16:1ω7 and 18:1ω7 were the same as those of the acidophilic methanotrophs, whereas other *Methylosinus* species only have low levels of these fatty acids. Until now, no acid-tolerant members of the *Methylosinus-Methylocystis* group are known. We propose that strain 29 represents a new species within the *Methylosinus* group, and a new name *Methylosinus acidiphilus* sp. nov. is proposed. Strain 29 (= DSM 17628 = ATT BAA-1243) is the type strain.

introduction

Methanotrophs are aerobic methane-oxidizing bacteria that use methane as an energy and carbon source (Hanson & Hanson, 1996). The first step of the oxidation of methane is catalysed by the enzyme methane monooxygenase, of which two types are known: the particulate methane monooxygenase (pMMO) and the soluble methane monooxygenase (sMMO). Nearly all methanotrophs have pMMO, except members of the acidophilic *Methylocella* group (Dedysh *et al*., 2000; Dunfield *et al*., 2003; Dedysh *et al*., 2004).

The methanotrophs occur in three monophyletic lineages within the *Proteobacteria*. These lineages differ in their carbon assimilation pathway and the arrangement of intracellular membranes (ICM), (Hanson & Hanson, 1996; Dedysh *et al*., 1998a; Dedysh *et al*., 1998b; Dedysh *et al*., 2000; Dedysh *et al*., 2002; Dunfield *et al*., 2003; Dedysh *et al*., 2004). The first lineage comprises type-I methanotrophs, which use the ribulose monophosphate pathway for formaldehyde fixation (*gammaproteobacteria*) and their ICM are arranged as bundles of disc-shaped vesicles distributed throughout the cell. The second and third lineages comprise type-II methanotrophs, which use the serine pathway for formaldehyde

fixation *(alphaproteobacteria*) and their ICM are arranged as paired membranes running along the periphery of the cell. These lineages include the *Methylocystis-Methylosinus* genera and the acidophilic methanotrophs, consisting of the genera, *Methylocella* and *Methylocapsa*. Members of these two latter genera are so far the only methanotrophs able to grow at a pH lower than 5.5. The isolation of these genera was possible only recently using new enrichment media with low salt content. Until now, acidophilic methanotrophs have been isolated from acidic peat bogs and acidic forest soils.

There are some reports where other type-II methanotrophs were found in acid environments. *Methylocystis* species have been detected by fluorescence *in situ* hybridisation (FISH) (Dedysh *et al*., 2003) and one neutrophilic strain of *Methylocystis* has been isolated from a *Sphagnum* peat bog (Heyer *et al*., 2002). Heyer and Suckow (1985) also describe the isolation of a neutrophilic bacterium of the genus *Methylosinus* from an acidic peat lake. This isolate was obtained in neutral medium and the optimum pH was not determined. Since most *Methylosinus* species are able to form exospores, survival under acid conditions may be possible for longer times (Dedysh *et al*., 1998a; Hanson *et al*., 1991).

The present study describes the isolation of strain 29 from a *Sphagnum* peat bog at Mariapeel, the Netherlands using the same medium, which was used to isolate acidophilic methanotrophs. This acidic peat bog was dominated by the peat moss *Sphagnum cuspidatum,* growing submerged below the water table. Previously, we showed that *S. cuspidatum* was colonized by methane oxidizing bacteria related to *Methylocella* (Raghoebarsing *et al*., 2005). Here we described the isolation of an unrelated methanotroph from these mosses, strain 29. As far as we know, strain 29 is the first acid-tolerant *Methylosinus* species, only distantly related to the presently known genera of acidophilic methanotrophs.

methods

sampling and culture conditions

Samples were collected from an acidic *Sphagnum* peat bog at Mariapeel nature reserve (the Netherlands; 51°24’90’’N; 5°54’90’’E). The samples (5 grams (w/v) of thoroughly washed *Sphagnum* mosses) were cut into small pieces and incubated submerged in a Fernbach flask with 1000 ml filter-sterile bog water pH 4.4. The Fernbach flask was closed with a screw cap and rubber septum. Methane was added aseptically to achieve a 10% concentration in the headspace. The flask was then incubated on a table shaker (150 rpm) at room temperature. After one week of incubation growth was detected by methane

consumption and turbidity of the culture. The bog water, enriched with methanotrophs, was used to make culture dilution series. Dilutions (10^{-2}-10^{-12}) were made in 120 ml serum cap bottles containing medium M2 with low nitrate content (Dedysh *et al*., 1998a) with a final pH of 5.0. After inoculation, the bottles were sealed with butyl rubber septa and methane was added aseptically to achieve a 15-20% concentration in the headspace (headspace: liquid-space ratio of 4:1). Bottles were incubated on a rotary shaker (100-200 rpm) at 25°C and the active methanotrophic dilutions were sub-cultured at 1-2 week intervals. The highest active methanotrophic dilution (10^{-8}) from the *Sphagnum* peat bog was plated on M2 agarose plates. After 2 weeks of incubation, small white colonies became visible. Repetitive picking and restreaking of these colonies resulted in a pure culture, strain 29. Purity of the culture was checked by plating on Luria-Bertani (LB) agar and by fluorescence *in situ* hybridization (FISH).

physiological tests

To test the pH range for activity and growth of strain 29, batch experiments with 10 mM of potassium buffered M2 media were adjusted to acidities 3.5 to 7. Two 120 ml serum bottles at each pH were inoculated with an active pre-culture of strain 29. The bottles were sealed with butyl rubber septa and 10 % (v/v) methane was added. After 5 days of incubation at room temperature, culture turbidity and methane consumption were analyzed.

carbon and nitrogen sources

Methanol (0.5% v/v), acetate (0.1% (w/v) and formate (0.1% w/v) were the possible carbon sources examined to determine the range of substrates utilized for growth and energy by strain 29. Nitrogen sources were also tested using M2 media with or without KNO_3 and $(NH_4)_2SO_4$. Growth was examined after 1 week of incubation.

analytical techniques

Methane (0.5 ml) was measured on a Hewlett-Packard model 5890 gas chromatograph equipped with a flame-ionization detector and a Porapak Q column (80/100 mesh). Turbidity as a measure of growth was analyzed on an Ultraspect K spectrophotometer at 600 nm.

PCR amplification of the 16S rRNA gene, the *pmoA* and *mmoX* genes

Chromosomal DNA from the methanotrophic isolate was extracted and the 16S rRNA gene was amplified with general bacterial primers 616F and 630R (Juretschko *et al*., 1998), which resulted in a PCR fragment of 1500 bp. Both the *pmoA* and the mmoX genes were amplified with general primers respectively (Holmes *et al*., 1995 and Aumann *et al*., 2000). Cloning, sequencing and phylogenetic analysis were performed according to Purkhold *et al*. (2000).

probe design, fluorescence *in situ* hybridization (FISH), microscopy

Probe S-S-Msina29-0218-a-A-22 (5'- GGG CCG ATC TTT CGG CAA TAA A- 3') was designed using the probe design tool of the ARB package (Strunk & Ludwig, 1996). Cells of strain 29 and fresh *S. cuspidatum* leaves and stem sections were used for FISH. Hybridizations with 20% formamide concentrations were performed as described previously (Amann *et al*., 1995). The optimum formamide concentration was determined by hybridization at concentrations varying 10-40 % formamide. Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescien-N-hydroxysuccinimide ester (Fluos) labeled derivatives from Thermohybaid (Ulm, Germany). For image acquisitions a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Jena, Germany) was used together with the standard software package delivered with the instrument (version 3.1).

electron microscopy

For Transmission Electron Microscopy, actively growing cells were collected by centrifugation, embedded in 1% water agar and fixed in 1% OsO_4/2% glutaraldehyde in 50 mM cacodylate buffer (pH 6.5) for 1 h at 4°C. After dehydration in an ethanol series, the samples were embedded in Spurr epoxy resin. Thin sections were cut on a Sorvall MT-5000 Ultra Microtome, stained with 2% (w/v) uranyl acetate in water and then post-stained with lead citrate (Reynolds, 1963). The specimen samples were examined with a JEOL JEM 100 CX-II transmission electron microscope.

Cryo-Scanning Electron Microscopy (Cryo-SEM) was performed on active batch cultures to examine cell morphology. A stub with a droplet of culture was frozen in liquid nitrogen. The sample was transferred in a transfer holder under vacuum at liquid-nitrogen temperature to the cold stage at -95°C into a cryo-preparation chamber CT 1500 HF (Oxford Instruments, High Wycomb, UK). The specimen was sputter-coated with 5 nm Pt and conveyed under high vacuum to the cold stage of a scanning electron microscope equipped with a cold-field emission electron gun (JSM 6300F; JEOL, Tokyo, Japan),

analysed and recorded at -180°C using a 5-kV accelerating voltage (Verhoeven *et al*., 2005).

lipid analysis

Cells were freeze-dried and extracted ultrasonically 5 times with dichloromethane (DCM)/methanol (2:1 v/v). An aliquot of the obtained total extract (TE) was methylated with diazomethane, silylated with N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). An internal standard (6,6-d2-3-methylheneicosane) was added prior to analysis. A second aliquot of the TE was treated with periodic acid and sodium borohydride according to procedure 2 described by Rohmer *et al*. (1984). The obtained fraction was silylated with BSTFA and analysed by GC and GC/MS, an internal standard was added prior to analysis. A third aliquot of the TE was hydrolysed with 2M HCl/MeOH (1:1 v/v) by refluxing for 4 h. After neutralizing with KOH and extraction with DCM, the extract was methylated, silylated and analysed by GC and GC/MS, an internal standard was added prior to analysis. In order to determine the double-bond position of the unsaturated fatty acids, this fraction was subsequently treated with dimethyl disulfide (DMDS) and iodine according to Carlson *et al.* (1989) and analysed by GC and GC/MS. The double bond position was assessed from the mass spectral fragmentation pattern of the DMDS-derivatized fatty acids.

nucleotide accession numbers

The sequences of the 16S rRNA gene (1,500 bp), the *pmoA* gene (500 bp) and the *mmoX* gene (1,200 bp) of strain 29 were deposited in the GenBank database under accession no. DQ076754, DQ076755, DQ076756.

results

Submerged *Sphagnum* mosses, the dominant plants in the peat bog at Mariapeel oxidize methane at high rate (Raghoebarsing *et al*., 2005). This oxidation is catalyzed by methanotrophs present on and in the submerged mosses. The methanotrophs from these mosses were enriched in a Fernbach flask with shredded plants in bog water. From this enrichment dilution series were made in mineral medium M2 with low nitrate content (pH 5.0) as was described for other acidophilic methanotrophs (Dedysh *et al*., 1998a). Plating on agarose plates resulted in a pure, active culture, designated strain 29.

In duplicate batch experiments at a range of acidities (3.5 to 7), the pH optimum for activity and growth of strain 29 was analyzed. Cell growth and methane consumption occurred at pH values between 4.5 and 6.4, with an optimum at pH 5.4 (Figure 1).

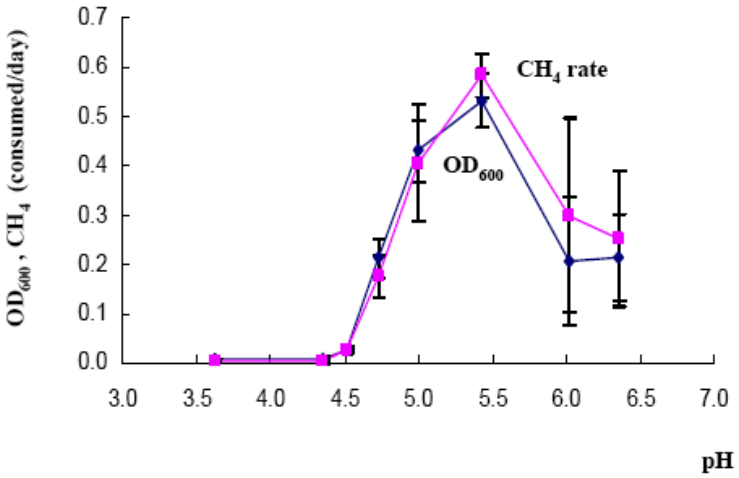


Figure 1: Acid tolerance of strain 29 as shown by optical density (600 nm) of the cell culture and methane consumption after 5 days as a function of pH

On plates, one-week-old colonies of strain 29 were small and white. Cells of strain 29 were Gram-negative, motile, sinus-shaped cells (Figure 2a, b). Strain purity was confirmed by repetitive plating on selective media (M2 and LB). On LB plates growth of strain 29 did not occur.

Strain 29 was capable of growth on methane and methanol as carbon and energy source. Growth did not occur on acetate and formate. Strain 29 utilized ammonium salts and nitrate as nitrogen sources. It was not able to fix atmospheric nitrogen when growing in nitrogen-free media.

Transmission electron microscopy (TEM) of cells from strain 29 showed that the arrangement of the intracellular membranes (ICM) in strain 29 corresponded to that of type-II methanotrophs, with paired membranes aligned to the periphery of the cell (Figure 2c). Both TEM and phase contrast microscopy showed the presence of exospores in cell cultures of strain 29.

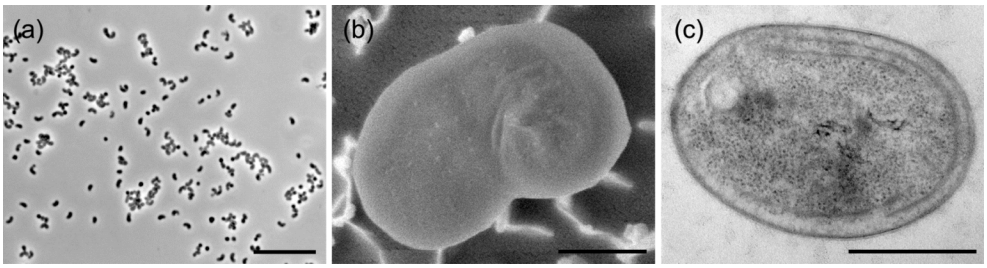
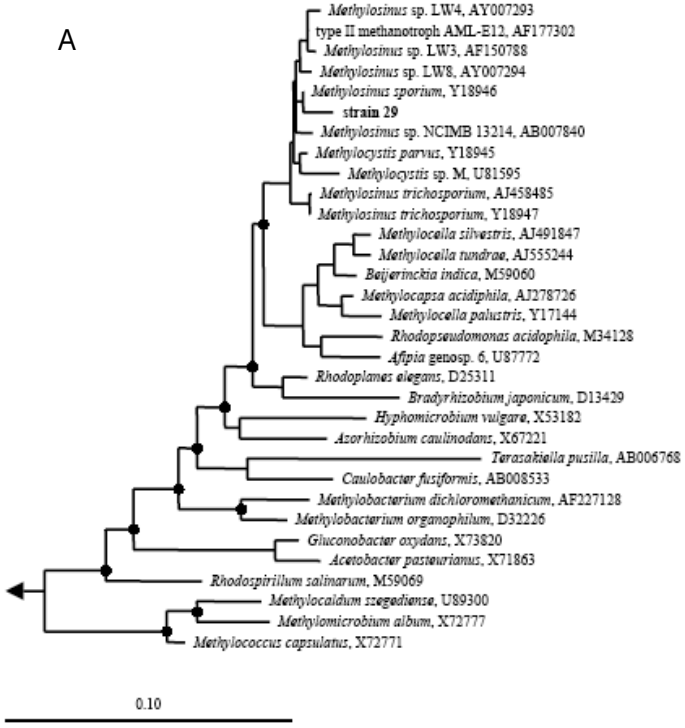


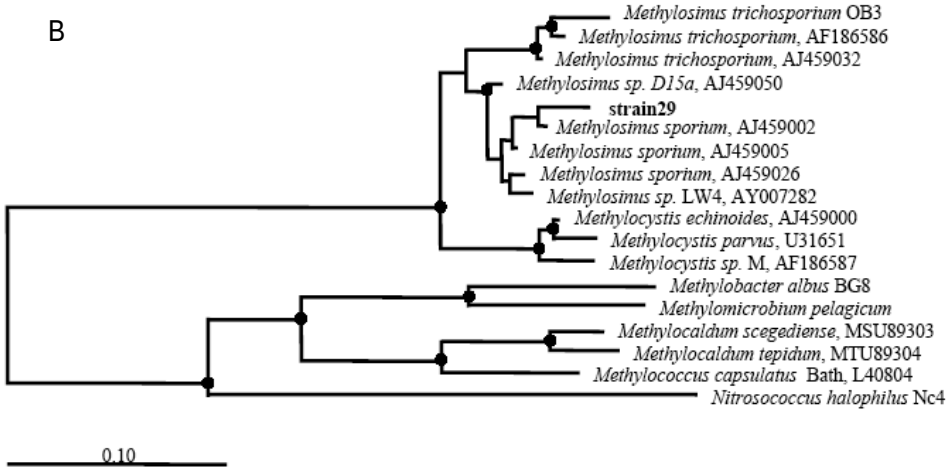
Figure 2: (a) Phase-contrast micrograph of cells of strain 29; bar, 10 µm. (b) Scanning electron micrograph of actively growing cells of strain 29; bar, 0.5 µm. (c) Transmission electron micrograph showing a section of cell of strain 29; bar, 0.5 µm

Phylogenetic analysis of the complete 16S rRNA gene showed that strain 29 was affiliated to the *Methylosinus-Methylocystis* cluster of type-II methanotrophs, within the *alphaproteobacteria*. It was most closely related to the 16S rRNA gene (Y18946) of the methane-oxidizing bacterium *Methylosinus sporium*, with a similarity of 98.6% (Figure 3a). The 16S rRNA gene sequence was used to design a probe (designated S-S-Msina29-0218-a-A-22) for fluorescence *in situ* hybridisation (FISH). This probe was combined with the *Bacteria*-specific probe mix EUB338 (Daims *et al*., 1999) and the group-specific probe for *alphaproteobacteria* ALF968 in a nested approach (for probe sequences see probeBase, Loy *et al*., 2003). All three probes hybridized with all cells from strain 29 (Figure 4a, b). No hybridization with the specific probe for strain 29 occurred with cells of *Beijerinckia indica* subsp. *indica*. FISH on the leaves and stem sections from *S. cuspidatum* showed that the abundance of cells of strain 29 was around 10% of the total bacterial population and located preferably on the stem leaves (Figure 4c). Phylogenetic analysis based on the complete *pmoA* and *mmoX* sequences indicated that *Methylosinus sporium* and *Methylosinus sp.* D15a were the closest neighbours of strain 29 (Figure 3b, c).

A


Methylosinus sp. LW4, AY007293
type II methanotroph AML-E12, AF177302
Methylosinus sp. LW3, AF150788
Methylosinus sp. LW8, AY007294
Methylosinus sporium, Y18946
strain 29
Methylosinus sp. NCIMB 13214, AB007840
Methylocystis parvus, Y18945
Methylocystis sp. M, U81595
Methylosinus trichosporium, AJ458485
Methylosinus trichosporium, Y18947
Methylocella silvestris, AJ491847
Methylocella tundrae, AJ555244
Beijerinckia indica, M59060
Methylocapsa acidiphila, AJ278726
Methylocella palustris, Y17144
Rhodopseudomonas acidophila, M34128
Afipia genosp. 6, U87772
Rhodoplanes elegans, D25311
Bradyrhizobium japonicum, D13429
Hyphomicrobium vulgare, X53182
Azorhizobium caulinodans, X67221
Tarasakiella pusilla, AB006768
Caulobacter fusiformis, AB008533
Methylobacterium dichloromethanicum, AF227128
Methylobacterium organophilum, D32226
Gluconobacter oxydans, X73820
Acetobacter pasteurianus, X71863
Rhodospirillum salinarum, M59069
Methylocaldum szegediense, U89300
Methylomicrobium album, X72777
Methylococcus capsulatus, X72771
0.10

B


Methylosinus trichosporium OB3
Methylosinus trichosporium, AF186586
Methylosinus trichosporium, AJ459032
Methylosinus sp. D15a, AJ459050
strain29
Methylosinus sporium, AJ459002
Methylosinus sporium, AJ459005
Methylosinus sporium, AJ459026
Methylosinus sp. LW4, AY007282
Methylocystis echinoides, AJ459000
Methylocystis parvus, U31651
Methylocystis sp. M, AF186587
Methylobacter albus BG8
Methylomicrobium pelagicum
Methylocaldum scegediense, MSU89303
Methylocaldum tepidum, MTU89304
Methylococcus capsulatus Bath, L40804
Nitrosococcus halophilus Nc4
0.10

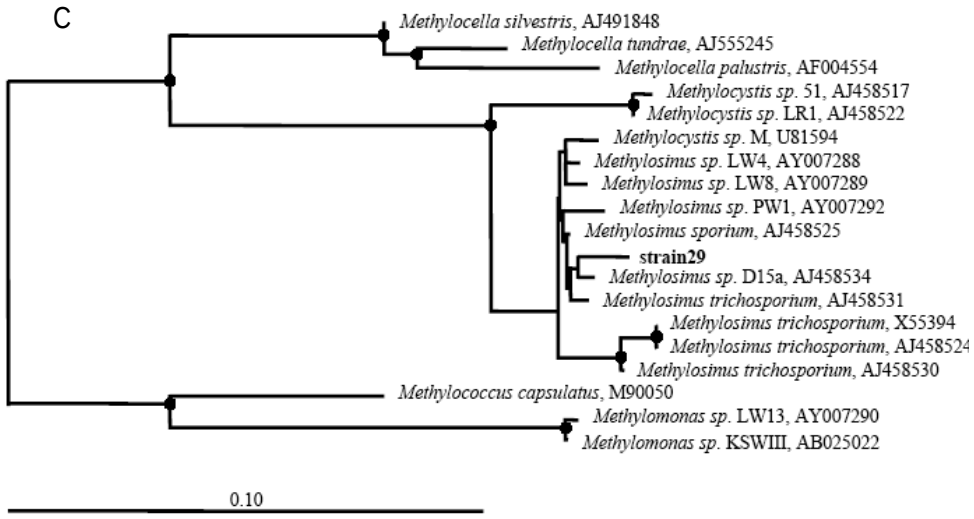


Figure 3: Phylogenetic trees indicating the position of strain 29. (a) 16S rRNA phylogeny. The tree was based on maximum likelihood analysis. The tree topology was confirmed by neighbour joining and maximum parsimony analyses. The bar represents 10% estimated sequence divergence. (b) and (c). Phylogenetic trees constructed based on the deduced amino acid sequences of the pmoA gene sequences (b) and mmoX gene sequences (c). The trees were calculated with the Fitsch-Margoliash algorithm of the PHYLIP package. The tree topology was confirmed by protein maximum likelihood analysis implemented in the ARB program package. The bar represents 10% estimated sequence divergence.

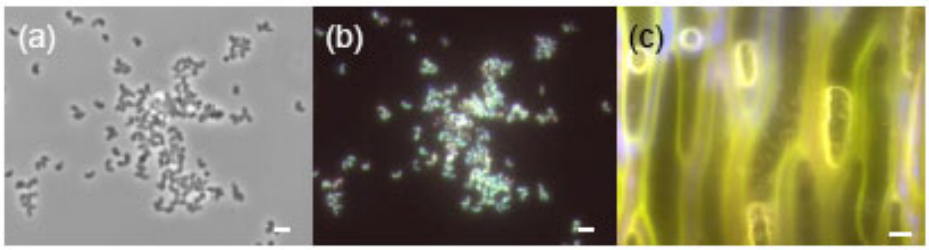


Figure 4: (a) Fluorescence *in situ* hybridisation of strain 29 with the newly developed specific rRNA-targeted oligonucleotide probe (labeled with Cy3, red), the Bacteria-specific EUB probe mix (Fluos, green) and ALF968 (specific for *alphaproteobacteria*, Cy5, blue). Strain 29 cells appear white, because of overlapping labels; bar, 2 µm. (b) FISH on a stemleave of *S. cuspidatum* hybridised with the same probes as in (a), bar, 5 µm. (see page 102 for color figure).

The major fatty acids of strain 29 were 16:1ω7 and 18:1ω7 (Table 1). Their double bond positions were established by DMDS derivatization. These fatty acids were previously shown to be the major fatty acids of the acidophilic methanotrophs *Methylocapsa acidophila* and the genus *Methylocella* (Dedysh *et al*., 2002; Dunfield *et al*., 2003; Dedysh *et al*., 2004). But they are not the major fatty acids of the genera *Methylosinus* and *Methylocystis* (Table 1). The characteristic fatty acid of those genera is 18:1ω8c. Apart

from these fatty acids, strain 29 also possessed the hopanoids diplopterol and 2-methyldiplopterol. These were detected as free alcohols. 2-Methyldiplopterol was previously reported to occur in type-II methanotrophs (Summons & Jahnke, 1992). Periodic acid treatment of the total extract resulted in the formation of bishomohopanol and in substantially smaller amounts of homohopanol, revealing the presence of bacteriohopanetetrol and bacteriohopanepentol derivatives, respectively, which are characteristic for many methylotrophs (Rohmer *et al.*, 1992).

Table 1: Cellular fatty acid composition (%) of the acidophilic methanotrophs (Dedysh *et al.*, 2004), *Methylosinus*/*Methylocystis* spp. (Bowman *et al.*, 1993) and strain 29.

Fatty acids	*Methylocella palustris* K^T	*Methylocella silvestris* $BL2^T$	*Methylocella tundrae* ($T4^T$, TCh1 & TY1)	*Methylocapsa acidiphila* $B2^T$	*Methyosinus*/ *Methylocystis* spp.	Strain 29
16:1ω7*	12.6	8.8	7.2-11.3	4.7	0.3-14.2	18.8
16:0	5.9	3.0	7.2-7.7	7.3	0.7-5.1	1.0
18:1 ω7*	78.6	82.2	59.2-61.7	78.3	14.8-42.3	78.4
18:0	0.9	1.2	0.4-0.6	7.6	0-5.0	1.8
18: 1 ω8c	0.0	0.0	0.0	0.0	52.9-73.6	0.0

*Mixture of ω7c/ ω7t

discussion

This study describes the isolation and characterization of the first acid-tolerant *Methylosinus* species from an acidic *Sphagnum* peat bog in the Netherlands. So far, acidophilic methanotrophs have been classified into two genera, *Methylocella* and *Methylocapsa*, isolated from various acidic environments such as peat bogs and forest soils. These two genera form a distinct subcluster within the type-II *Methylosinus-Methylocystis* cluster (Figure 3a). Isolation of these acidophilic methanotrophs had become possible using media with low salt content (Dedysh *et al.*, 1998b).

In our previous paper (Raghoebarsing *et al.*, 2005) we performed a full cycle rRNA gene analyses on *Sphagnum* material and found 16S rRNA gene sequences closely related to the genera *Methylocella-Methylocapsa*. For the isolation procedure of strain 29 from submerged *Sphagnum*, we used a medium, which was previously used to isolate other acidophilic methanotrophs. Therefore, we expected to find an acidophilic methanotroph

belonging to genera *Methylocella* or *Methylocapsa*. Surprisingly, using the same medium, our isolation resulted in the isolation of a bacterium from the genus *Methylosinus*. An explanation could be that the medium M2 might have missed unknown components, which were present in the bogwater from the Mariapeel. Also, since in the Mariapeel there was a tight association between the dominant *Methylocella* related sp. and the *Sphagnum* mosses, these bacteria might be depending on plant derived growth factors.

In acidic *Sphagnum* peatlands from Siberia and northern Germany, Dedysh *et al*. (2003) found predominantly type-II methanotrophs using specific fluorescence *in situ* hybridization (FISH). Most type-II methanotrophs observed by FISH belonged to the *Methylocystis* subgroup and not to the *Methylocella*/*Methylocapsa* subgroup. This was surprising since no acidophiles of the genera *Methylocystis* and *Methylosinus* have ever been isolated. However, there are reports describing the isolation of one neutrophilic strain of *Methylocystis* from a *Sphagnum* peat bog (Heyer *et al*., 2002) and the isolation of one neutrophilic bacterium of the genus *Methylosinus* from an acidic peat lake (Heyer & Suckow, 1985).

Our study describes the first isolation of an acid tolerant type-II methanotroph of the *Methylocystis-Methylosinus* subgroup. This bacterium was acid tolerant and capable of growth below pH 5.5. The 16S rRNA gene sequence of strain 29 clustered within the *Methylosinus* group (Figure 3a). Also the *pmoA* and *mmoX* genes of strain 29 clustered within the *Methylosinus* group (Figure 3b, c). FISH on fixed cells of strain 29 also showed that all cells hybridised with probe S-S-Msina29-0218-a-A-22. We also performed FISH on stem leaves and stem sections from submerged *Sphagnum* mosses to determine the ecological habitat of strain 29. Indeed, we could detect cells of strain 29 in these mosses. The abundance was around 10%.

We have, thus, isolated the first acidophilic member of the *Methylocystis-Methylosinus* subcluster of the type-II methanotrophs. It should be noted, however, that this isolate can not explain the high numbers of bacteria in the *Methylocystis-Methylosinus* subcluster in Russian and German acidic peatlands (Dedysh *et al*., 2003) since their FISH analyses indicated the absence of *Methylosinus sporium*, a bacterium phylogenetically closely related to strain 29 (Figure 3).

The phospholipid fatty acids (PLFA) 16:1ω7 and 18:1ω7 were the major fatty acids in strain 29 (Table 1) but are not the major fatty acids in other *Methylosinus* species (Bowman *et al*., 1993). Dedysh *et al*. (2002) showed that the PLFA 16:1ω7 and 18:1ω7 are a characteristic feature of acidophilic methanotrophs. This could indicate that these fatty acids may play a role in growth in acidic environments. The PLFA component 18:1ω8c, which is highly characteristic for the *Methylosinus-Methylocystis* group, was not found in strain 29 (Table 1). The PLFAs of strain 29 suggested some similarity to the acidophilic methanotrophs *Methylocella* and *Methylocapsa*, molecular 16S rRNA, *pmoA* and *mmoX* data, did show that this was not the case. Until now, analysis of PLFAs provided useful fingerprints for taxonomy and identification of methanotrophs (Hanson & Hanson, 1996). Our findings suggest that identification of methanotrophs on basis of PLFAs should be reconsidered. Apparently, fatty acid composition is determined more by environmental conditions than taxonomic positions.

description of *Methylosinus acidiphilus* sp. nov.

Methylosinus acidiphilus (L. adj. acidus, sour, N.L. neut. N. acidum, acid; Gr. adj. *Philos*, loving; N.L. masc.adj. *acidiphilus,* acid-loving).

Description as for the genus plus the following traits. Growth occurs between 20-25°C and at pH values 4.5-6.5. The type strain is strain 29, which was isolated from an acidic *Sphagnum* peat bog, the Netherlands and is deposited as strain DSM 17628 and ATCC BAA-1243, respectively.

acknowledgements

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chapter 4

microbial diversity in two *Sphagnum* peat bogs

abstract

Peatlands are important in the global carbon cycle. The microbial diversity of microbes inhabiting two Dutch *Sphagnum* peat ecosystems was analyzed using complementary molecular methods. The first peat system, the Mariapeel, was analyzed in two successive years. Comparison of the 16S rRNA gene sequences revealed that the microbial diversity in the two years was very similar. The 16S rRNA gene libraries were dominated by representatives of the *Proteobacteria*, *Acidobacteria*, *Planctomycetes*, *Actinobacteria* and unclassified clones. The second peat system, the Wierdense Veld, contained the same dominant phylogenetic groups as the Mariapeel. The 16S rRNA gene sequences of the three major groups, the alpha type II methanotrophs, the *Acidobacteria/Holophaga* and the *Planctomycetes* were used to design specific oligonucleotide probes for FISH. *In situ* hybrizations showed a numerical dominance of the methanotrophs, and a high morphological diversity of the *Planctomycetes* inside the *Sphagnum* mosses. Hybridization of the *Acidobacteria/Holophaga* probes gave only very faint signals. The morphological diversity of microorganisms in and on the *Sphagnum* mosses was confirmed by thin section electron microscopy. In addition, a clone library using *Planctomycetes* specific PCR primers was made from the mosses of the Mariapeel. The retrieved sequences confirmed the high abundance and diversity of *Planctomycetes*.

introduction

Peatlands (bogs and fens) are important ecosystems in the global carbon cycle and cover 2-3% (3.36×10^6 ha) of terrestrial landmass (Fisher, 1998). They are known as regions of long-term carbon storage and contain approximately one-third of the world's carbon (Gorham, 1991). Being important terrestrial sinks or sources of carbon, peat bogs may influence global carbon cycling (Gorham 1991; Yavitt *et al.*, 1997). Peat bogs are ombrotrophic (only fed by rainwater and dry atmospheric decomposition), acidic and dominated by peat mosses from the genera *Sphagnum*. In these systems, methane (CH_4), produced by methanogenic Archaea, and carbon dioxide (CO_2) are the end-products of anaerobic degradation of organic matter.

The emission of methane from peat bogs is by far lower than the production in the bog, and methanotrophic bacteria play an important role in this observation. Methanotrophic bacteria are methane-oxidizing bacteria which convert CH_4 into CO_2 and cell carbon (Hanson & Hanson, 1996). In peat bogs, these bacteria limit the release of CH_4 into the atmosphere (Dedysh *et al.*, 1998; Raghoebarsing *et al.*, 2005) and may even act as sink for atmospheric CH_4 (Bridgeham & Richardson, 1992; Gorham, 1991;Yavitt *et al.*, 1997).

Recently, we found that in a *Sphagnum* bog in the Netherlands, methane-oxidizing bacteria live in symbioses with *Sphagnum* mosses (Raghoebarsing *et al.*, 2005). The methanotrophic bacteria living in and on cells of *Sphagnum cuspidatum* oxidize CH_4 into CO_2, which in turn is used by the mosses for their photosynthesis. Evidence for this process was based on molecular and ^{13}C-labelled bacterial and plant biomarker studies. Isolation of these endophytic methanotrophic bacteria was not yet successful. Studies by Basiliko *et al.* (2004) suggested that methane consumption can occur inside the *Sphagnum* mosses but the presence of methanotrophic bacteria was not investigated. Until now, only acidophilic methanotrophic bacteria from the genera *Methylocella* and *Methylocapsa*, were isolated from peat-cores in Northern peatlands (Dedysh *et al.*, 2002; Dedysh *et al.*, 2000; Dedysh *et al.* 1998). Bryophytes such as *Sphagnum* mosses may be unique host plants for microorganisms but the diversity has not well been characterized yet (Opelt & Berg, 2004). Barkovskii & Fukui (2004) developed a new method to isolate peat microorganisms. Their method was based on isolation by sequential elution of peat and molecular analysis such as terminal-restriction fragment polymorphism (T-RFLP). The microorganisms isolated were all *Actinobacteria*, but the T-RFLP analysis show a much higher taxonomic diversity of microorganisms. This research illustrates that isolation in these systems is very difficult as usual and that molecular methods give a different view of the microbial diversity.

The microbial diversity in peat bogs has so far focussed on methanogenic Archaea and methanotrophic bacteria (Juottonen *et al.*, 2005). The studies performed so far target different parts of the peat bogs and use different molecular techniques. One study aimed to characterize the biodiversity of the microorganisms associated with *Sphagnum* mosses using two cultivation-independent techniques, denaturing gradient gel electrophoresis (DGGE) and single-strand conformation polymorphism (SSCP), (Opelt & Berg, 2004). The second focused on the microbial diversity in peat samples (peat-core) using fluorescence *in situ* hybridization (FISH), (Pankratov *et al.*, 2005). Both studies showed the presence of different bacteria phyla, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Acidobacteria* and *Planctomycetes* in the peat ecosystem. Especially the detection of *Planctomycetes* is remarkable and coincides with the emerging view of their unique genetic repertoire in C_1-genes (Chistoserdova *et al.*, 2004).

In the Netherlands peat bogs have practically disappeared due to drainage, reclamation and peat harvesting. At present only small relics are left which can be characterized as desiccated peat remnants. As typical peat bog vegetation has become very rare, major efforts are dedicated to the rehabilitation of these systems (Smolders *et al.*, 2003). The

first step in restoring ombrotrophic bogs is the rewetting of bog remnants which usually results in an increased growth of *Sphagnum cuspidatum*. At present, a number of bog remnants in the Netherlands have been successfully rewetted and many studies have been undertaken to characterize the hydrology and chemistry of these systems (Tomassen *et al*., 2003). Compared to undisturbed ombrotrophic bogs, many of the bog remnants appear to be rich in nutrients, nitrogen and phosphorus and also in carbon dioxide and methane. Until now, however, an insight in the microbial diversity of rewetted bog remnants is lacking.

The research presented here was performed to analyse the microbial community from two bog remnant in the Netherlands. The two peat systems were both rich in nutrients and the dominant moss species was *Sphagnum cuspidatum*. The microbial diversity of *Sphagnum cuspidatum* was analyzed using 16S rRNA gene clone libraries and FISH. Complementary to this approach transmission electron micrography (TEM) on *Sphagnum* sections and stem leaves was performed.

methods

sampling sites

Sphagnum cuspidatum peat mosses were collected from two ombrotrophic bogs, the Mariapeel (Limburg, the Netherlands: 51° 24' 90"N; 5° 54' 90" E) and the Wierdense Veld (Overijssel, the Netherlands: 52° 22' 55"N; 6° 31' 16" E).

nucleic acid extraction

Total genomic DNA from *S. cuspidatum* mosses with bacteria attached was isolated. The mosses, 5 g (w/w), were rinsed with demiwater to remove bacteria, which were loosely attached. Mosses were transferred to a mortar and liquid nitrogen was added. Frozen mosses were grinded till powder suspense was left over. The powder was transferred to a 50 ml tube and SDS and NaCl were added to final concentrations of 2% and 1M, respectively. To this mixture 1 volume of phenol-chloroform-isoamylalcohol (25:24:1) was added and incubated in a waterbath of 65°C for 2h. After incubation the mixture was centrifuged for 20 mins at 4000 rpm. The water phase obtained after centrifugation was transferred to a new tube and 1 volume of chloroform-isoamylalcohol (24:1) was added, mixed and centrifuged for 20 mins at 4000 rpm. The aqueous phase was transferred to a new tube again. Genomic DNA was precipitated by adding 0.1 volume of 3M NaAc (pH 4.8), 2 volumes of 100% ethanol. After an incubation of 30 mins at -20°C, it was centrifuged for 20 mins at -20°C. The resulting gDNA pellet was washed with 70% ethanol to remove

additional salts, dried and dissolved in 0.5 ml Tris-HCl/EDTA (TE) buffer (pH 8.0). The gDNA solution was purified by incubating the DNA solution with Rnase stock solution (10µg/µl) for 15 mins at 37°C. Then 500 µl of Sepha-glass bead suspension of the FlexiPrep Kit (Pharmacia P-L Biochemicals Inc.) was added. The mixture was mixed well and incubated for 1 min at RT. The glass beads with the bound DNA were pelleted by centrifugation (30s; 10,000× g) and washed two times in 200 µl of washing buffer of the FlexiPrep Kit and finally once in 300 µl of 70% ethanol. After the removal of the ethanol the glass bead/DNA pellet was dried for 10 mins at RT. To dissolve the DNA attached to the glass beads, 50 µl of distilled water was added. Glass beads were removed by centrifugation and the gDNA was stored at 4°C.

PCR amplification & 16S rRNA gene sequencing

The gDNA was a mixture of moss (plant) DNA and bacterial DNA. To differentiate between those a PCR was performed with general bacterial 16S rRNA gene primers. The primers used for amplification were 616F & 630R (Juretschko *et al*., 1998), resulting in a PCR product of approximately 1500 bp. A separate PCR was performed with a *Planctomycetales* specific primer, Pla46 (Neef *et al*., 1998) and the universal primer 1390R (Zheng *et al*., 1996). This was necessary because the abundance of *Planctomycetes* in clone libraries using the general primers is mostly underestimated (Schmid *et al*., 2005). PCR amplifications were performed with a T gradient thermal cycler (Biometra, Germany) and, subsequently the 16S rRNA gene amplifications were cloned directly using the TOPO TA cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Plasmid-DNA was isolated with the FlexiPrep Kit (Amersham Pharmacia P-L Biochemicals Inc.). Plasmids with an insert of the expected size were identified by agarose (1.0%) gel electrophoresis after EcoRI digestion (5 U, EcoRI buffer for 3 h at 37 °C). Sequencing was done on a BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems, Foster City, CA). The reaction mixtures were analyzed with the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Partial 16S rRNA gene fragments were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site. The sampling effort in each clone library was evaluated by calculating the coverage C (Singleton *et al.,* 2001) according to the equation $C=[1-(n1/N)] \times 100\%$, where n is the number of OTUs representing only a single clone and N is the total number of clones analyzed.

phylogenetic analysis

From these clone libraries the sequences of the 16S rRNA gene (~600 bp) were compared with their closest relatives in the GenBank databases as determined using BLASTN searches (http://www.ncbi.nih.nlm.edu/BLAST) and analyzed using the ARB program package (Ludwig *et al.*, 2004). All 16S rRNA sequences were aligned automatically using the respective tool of the ARB package. Subsequently, the alignments were corrected by visual inspection. Phylogenetic analyses of 16S rRNA gene sequences were performed by applying neighbor-joining, ARB parsimony and maximum likelihood analysis, as well as Bootstraping analysis and chimera test according to Schmid *et al*. (2003).

probe design, fluorescence *in situ* hybridisation (FISH) & microscopy

The obtained 16S rRNA gene sequences of clones from the categories *Planctomycetes* and *Acidobacterium* were used to design new oligonucleotide probes using the probe design tool of the ARB program package (Ludwig *et al.*, 2004). For the *Planctomycetes*-related clones AR6 and AR14 the probes S-*-AR6&14-0286-a-A-18 (5'-ACG CTC GCA CGC CCG GTA-3') and S-*-AR6&14-0634-a-A-18 (5'-GGA CGG TCG TAT CGG CGC-3') were designed.

From the 16S rRNA gene sequences of the *Acidobacterium*/*Holophaga* group different probes were designed. Clone AR8 was used to design three probes specific for the *Acidobacterium* group, S-*-AR8POS220-0220-a-A-18 (5'-GCG GAC TCC TCT CTC AGC-3'); S-*-AR8POS292A-0292-a-A-18 (5'-TCA GTT CCA GTG TGT CCG T-3') and S-*-AR8POS292B-0220-a-A-18 (5'-TCA GTG CCA GTG TGG CCG T-3'). The clones AR49 and 179 were used to design one specific probe for the *Holophaga* group S-*-AR49&179HOLO-1420-a-A-18 (5'-CCT CTT CAA GTG CTC CCG-3'). These probes were used for hybridization with fresh stem sections of *S. cuspidatum* and stem leaves according to Raghoebarsing *et al*. (2005). Beside these specific probes we also used the general bacterial probe mix, consisting of EUB338, EUB338II and EUB338III as further described in probeBase (Loy *et al.*, 2003); probe ALF968 (specific for *alphaproteobacteria*); probe Pla46 (specific for *Planctomycetes*) and two probes (18ALF0218 & 18ALF1437) specific for acidophilic methanotrophs (Raghoebarsing *et al.*, 2005). Hybridizations were performed at 10, 15 and 20% formamide concentrations.

electron microscopy

For Transmission Electron Microscopy (TEM), both stems and leaves of *S. cuspidatum* were fixed in 1% OsO4/2% glutaraldehyde in 50 mM cacodylate buffer (pH 6.5) for 1 h at 4°C. After dehydration in an ethanol series, the samples were embedded in Spurr epoxy resin.

Thin sections were cut on a Sorvall MT-5000 Ultra Microtome, stained with 2% (w/v) uranyl acetate in water and then post-stained with lead citrate (Reynolds, 1963). The specimen samples were examined with a JEOL JEM 100 CX-II transmission electron microscope.

nucleotide sequence accession numbers

The nucleotide sequences of the 16S rRNA gene amplified from community *Sphagnum cuspidatum* DNA will be deposited into Genbank.

results

diversity of 16S rRNA gene sequences and phylogeny

The microbial diversity in submerged *Sphagnum cuspidatum* was investigated using a molecular approach. Total genomic DNA from *S. cuspidatum* mosses of the Mariapeel and Wierdense Veld was isolated and bacterial 16S ribosomal RNA genes were amplified, cloned into *E. coli*, sequenced and analyzed phylogenetically with the ARB software package. Two general bacterial clone libraries of the Mariapeel were generated. The first one was established in August 2001 (57 clones) and the second in April 2002 (49 clones). The general bacterial clone library of the Wierdense Veld was generated in July 2004 (57 clones). Partial 16S rRNA gene sequences (~600 bp) of all clones in the library were analyzed and compared with closest relatives in the GenBank databases. The survey for all clones from the three clone libraries showed a division in four major phylogenetic groups: 15-21 clones (26-43%) of *Proteobacteria*, dominated by *alphaproteobacteria*; 9-22 clones (18-39%) of *Acidobacteria/Holophaga*, 2-7 clones (4-14%) of *Planctomycetes*, a minor group of 1-4 clones (2-7%) of *Actinobacteria* and 9-17 (18-30%) of the clones were unclassified (Table 1). From each clone library the coverage was estimated using similarity matrixes and threshold values of 97% (OTUs). The first clone library of the Mariapeel (August 2001) had a coverage of 82% of the OTUs and the second clone library of the Mariapeel (April 2002) had a coverage of 65%. The total coverage of both these clone libraries together was 85%. The coverage of the Wierdense Veld (July 2004) was 67%.

Table 1: Blast results from the 16S rRNA genes sequences (~600 bp) from the general bacterial clone libraries of the Mariapeel and Wierdense Veld.

Phylogenetic group	**Mariapeel** (August 2001)	**Mariapeel** (April 2002)	**Wierdense Veld** (July 2004)
Proteobacteria	17	21	15
Acidobacteria/Holophaga	22	9	18
Planctomycetes	2	7	6
Actinobacteria	4	3	1
Unclassified clones	12	9	17
Total clones	**57**	**49**	**57**

Almost complete 16S rRNA gene sequences (~1500 bp) of 1-3 representatives of each phylogenetic group of every clone library were completely sequenced. The phylogenetic affiliation of the full length sequences was determined with the ARB software package and is shown in Figure 1. The major phylogenetic group in all three clone libraries is the type II methane-oxidizing bacteria within the *alpharoteobacteria*. Both ecosystems contained the same species. The second major phylogenetic group is the *Acidobacteria/Holophaga*. 16S rRNA gene sequences of this group are found widespread and few cultured species are known (Liesack *et al*., 1994; Ludwig *et al*., 1997).

The detection of *Planctomycetes* related clones as one of the phylogenetic groups in all three clone libraries suggested a possible involvement of these microorganisms in peat ecosystems. We investigated the *Planctomycetes* diversity in these bogs in more detail by constructing a clone library. The *Planctomycetes* clone library (56 clones) was generated from *S. cuspidatum* mosses from the Mariapeel (April 2002). The 16S rRNA gene sequences (~600 bp) obtained from this clone library were analyzed the same way as were the general bacterial clone libraries. The 56 clones were estimated to represent 88% of the OTUs in the *Planctomycetes* clone library. The phylogenetic analyis of representatives of these *Planctomycetes* related 16S rRNA gene sequences are shown in Figure 2. The majority of the clones were affiliated with known members of the *Planctomycetes* (*Gemmata*, *Nostocoida*, and *Planctomyces*). Remarkably, 5 clones clustered with the sister group of the *Verrucomicrobia* and 7 clones remained unclassified.

in situ detection (FISH)

The 16S rRNA gene sequences (1500 bp) of two *Planctomycete* related clones, AR6 and AR14, and the *Acidobacterium*/*Holophaga* related clones, AR49 & AR179, were used to design species-specific oligonucleotide probes for FISH. FISH was performed on cross sections of stems and on stem leaves from submerged *S. cuspidatum* and the species-specific probes were combined with the general bacterial probe EUBmix, the *alpha*p*roteobacteria* probe, the *Planctomycetes* probe (Pla16) and the previously designed methanotrophic probes (18ALF) (Raghoebarsing *et al.*, 2005). Hybridizations with the probe EUBmix and probe ALF968 showed that the *alphaproteobacteria* were the dominant group of bacteria in these ecosystems. About 75% of the *alphaproteobacteria* hybridized with the two species-specific methanotrophic probes. The bacteria were present inside the hyaline cells of the stem and on the stem leaves.

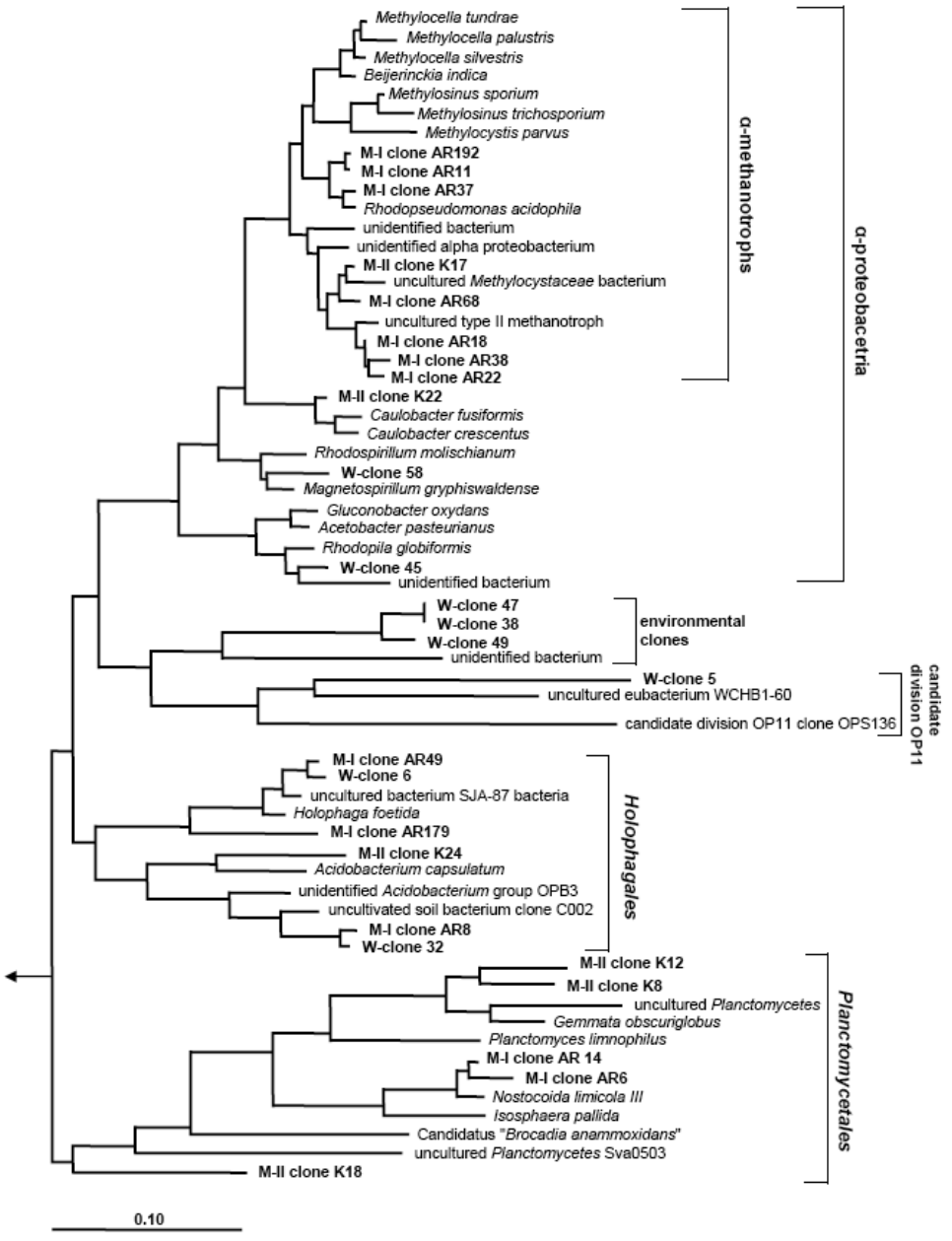

Figure 1: Phylogenetic 16S rRNA gene tree reflecting the relationship of the representative clones of the Mariapeel (August 2001, labeled M-I, April 2002, labeled M-II) and the Wierdense Veld (labeled W). The tree was constructed with full sequences by the maximum-likelihood method. The bar represents 10% sequence divergence.

Figure 2: 16S rRNA gene based phylogenetic tree indicating the position of 37 clones of the clone library from the Mariapeel (April 2002) obtained by a *Planctomycetes* specific PCR amplification. The tree was constructed by creating a scaffolding tree of full sequences by the maximum likelihood method and a subsequent adding of shorter sequences (~600 bp). The bar represents 10% estimated sequence divergence.

Hybridizations with the two newly designed specific *Planctomycetes* probes (AR6&14) and the bacterial probe EUBmix showed clearly that representatives of this group inhabited *S. cuspidatum* stem cells and stem leaves (Figure 3). This is consistent with their presence in the clone library. The morphology of the *Planctomycetes* was very diverse. Some of the cells hybridizing with the AR6&14-specific probes were coccoid, while other positive cells were arranged in chains. Hybridizations with the general *Planctomycetes* probe Pla46 gave only faint signals.

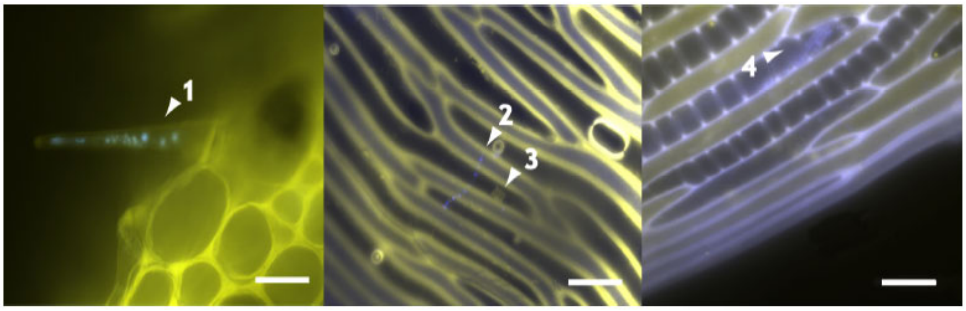

Figure 3: *In situ* detection of *Planctomycetales* related microorganisms in *S. cuspidatum*. Epifluorescence micrographs of the hybridization of the two specific probes and the general bacterial probe (a) with the stem. Coccoid cells (arrow 1) hybridizing with all tree probes are colored light blue, (b) with a stem leaf. Chain structures cells hybridizing with all tree probes are colored yellow (arrow 2) and sarcina-like cells hybridizing with the bacterial probe (arrow 3), (c) also with a stem leaf. Coccoid cells inside the hyaline cells hybridizing with all tree probes are colored light-blue (arrow 4). Scale bar, 10 µm. (see page 103 for color figure).

The *Acidobacterium* specific probes (AR8POS220, AR8POS292A and AR8POS292B) and the *Holophaga* specific probe (AR49&179HOLO) in combination with the general bacterial probe EUBmix were also applied. Positive signals could be obtained with the bacterial probe mix, but the *Acidobacterium*- and *Holophaga*-specific probes only gave very weak signals.

electron microscopy (TEM)

Complementary to the light microscopic analysis, the morphology from the microbial community inside and on the *Sphagnum* mosses was also analysed by electron microscopy. TEM pictures of the *Sphagnum* mosses showed a high morphological diversity of microorganisms inside the hyaline cells and on the stem leaves. The acidophilic methanotrophs were arranged in sarcina-like clusters as depicted by both TEM and FISH micrographs in Raghoebarsing *et al*. (2005). TEM showed several coccoid bacterial cells in *Sphagnum* stem and leaf sections. In these coccoid cells no compartmentalization or nucleoid bodies typical for *Planctomycetes* were observed (Lindsay *et al.*, 2001). TEM did show a high morphological diversity of bacterial cells. The diversity is based on various cell

shapes, structures and the arrangement of the cytoplasm and the location of the bacterial cells in and on *S. cuspidatum* cells (Figure 4). Intriguing was the observation of bacterial cells 'degrading' the plant cell walls (Figure 5). The high morphological diversity of the bacterial cells is consistent with the high diversity in both the clone library and the hybridization experiments.

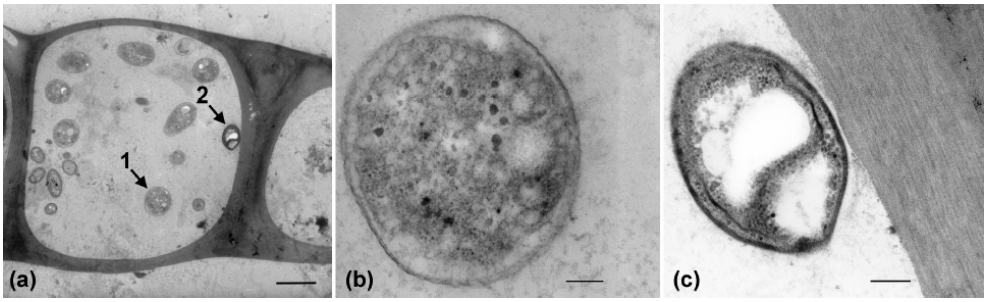

Figure 4: Transmission electron micrographs. (a) Overview of *S. cuspidatum* leaf cells with inside several bacterial cells. Scale bar, 2 µm. Detailed views of two different morphotypes of the bacterial cells are shown in (b) 1 and (c) 2. Scale bar, 0.2 µm.

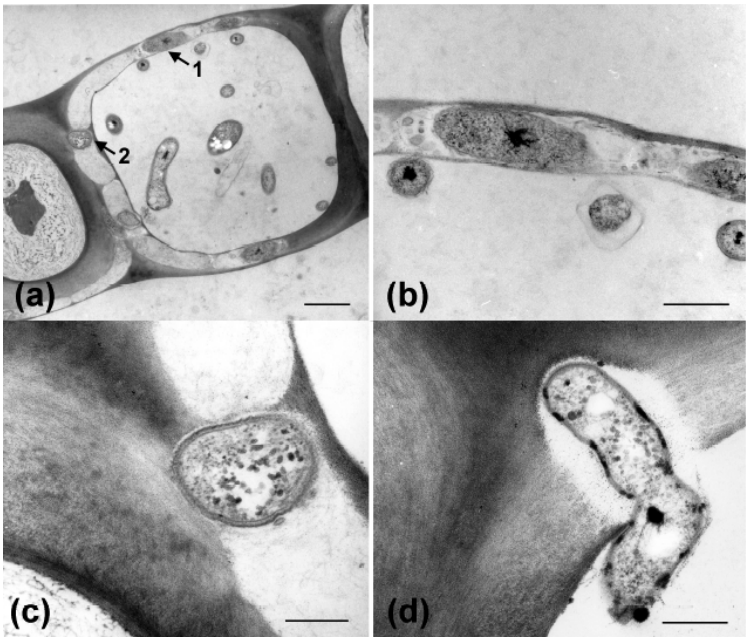

Figure 5: Transmission electron micrographs. (a) Cells of a *S. cuspidatum* leaf with bacterial cells "degrading" the cell wall and several bacterial cells inside. Scale bar, 2 µm. In (b), (c) and (d) detailed views of the morphological different bacterial "degrading" cells are shown. Scale bar, 0.5 µm.

discussion

The microbial community living in *Sphagnum cuspidatum* mosses in two bog remnants in the Netherlands was investigated. The characterization was based on molecular 16S rRNA gene analysis, *in situ* detection (FISH) and electron microscopy. Using these techniques we found a high species diversity of *S. cupidatum*-associated bacteria in these two bog remnants.

Prior to DNA isolation, the mosses were carefully rinsed with demineralized water to remove the loosely attached bacterial cells. Thus, only bacteria living in or closely attached to the mosses were studied. The reproducibility of the molecular methods was checked by performing the same procedure twice in successive years. In both years the 16S rRNA gene clones could be categorized in the same four major phylogenetic groups and the number of clones in each category was very similar. The same molecular approach was also applied to the *S. cupidatum* mosses from another peat remnant (Wierdense Veld). Again, the 16S rRNA clones could be categorized in the same four major phylogenetic groups and the numbers were comparable with those of the Mariapeel. This suggests a very similar microbial diversity in the *S. cuspidatum* mosses from these two bog remnants.

The dominance of acidiphilic type II methanotrophs was previously demonstrated for the Mariapeel (Raghoebarsing *et al.,* 2005). It was shown that these methanotrophic bacteria were living in symbioses with *S. cuspidatum*, providing a possible explanation for the efficient carbon cycling in the ecosystems. Analyzing the total microbial community in these mosses now revealed the coexistence of these methanotrophic bacteria (*alphaproteobacteria)* with other bacteria representing different phyla such as *Acidobacteria*, *Planctomycetes* and *Actinomycetes*. Especially the presence of *Planctomycetes* was intriguing because of their possible role in the conversion of one carbon compounds and formaldehyde detoxification (Bauer *et al*., 2004; Chistoserdova *et al*., 2004). *Planctomycetes* posses C_1 genes which are also present in methanogenic Archaea and methylotrophic *Proteobacteria.* The *Planctomycetes* specific clone library generated from the mosses of the Mariapeel, showed a high abundance and affiliation to known *Planctomycetes*.

The present analyses of 16S rRNA gene sequences related to *Nostocoida* species is remarkable since the species have only been observed in waste water treatment systems (Liu *et al*., 2001) in which they play a role in sludge foaming. The FISH analysis revealed a high morphological diversity of *Planctomycetes.* Some cells occurred as coccoid cells, while

others appeared in chains similar to the *Nostocoida limnicola* cells described in waste water (Liu *et al*., 2001). The possible role for *Planctomycetes* in these ecosystems could be addressed by using primers for functional genes of their C_1 metabolism after incubation with stable isotopes (i.e. $^{13}CH_3OH$) or radioisotopes (i.e $^{14}CH_4$ or $^{14}CH_3OH$) followed by FISH-microautoradiography (Kalyuzhnaya *et al*., 2005; Radajewski *et al*., 2002; Ginige *et al*., 2004). This approach would be complementary to the specific *Planctomycetes* primers used in our study. The retrieval of 5 *Verrucomicrobium* sequences and of 7 unclassified clones showed that the primer set (Pla46 and 1390R) is still not specific enough to amplify *Planctomycetes* 16S rRNA genes only. In our study we found that *Planctomycetes* contributed up to 14% to the obtained 16S rRNA gene sequences, which is higher than the 1% which was determined in 4 different Russian peat bogs (Pankratov *et al*., 2005) using the Pla46 probe. We also observed weak signals with the Pla46 probes compared to the newly designed specific *Planctomycetes* probes AR6&AR14 probes which gave much better signals.

Another important group of clones in the 3 libraries was composed of *Acidobacterium*/*Holophaga* members. Up to 40% of the clones were related to this group of bacteria for which very limited information is available. These bacteria have been implicated in the assimilation of C_1 compounds, especially in studies using methanol (Radajewski *et al*., 2002). The abundance of this group in *Sphagnum* mosses could not be estimated in FISH analysis because the probes used only gave very weak signals. Similar problems were also encountered by Pankratov *et al.* (2005) in the 4 Russian peat ecosystems where the two general *Acidobacterium*/*Holophaga* probes covered only 2% of the signal from the general bacterial probe. Future research will be necessary to elucidate the role of these bacteria in the peat ecosystems using a similar approach as outlined for the *Planctomycetes*. Taken together this study showed that the microbial diversity in *S. cuspidatum* is composed of 4 different groups. The role of the *Acidobacterium*/*Holophaga* and *Planctomycetes* should be addressed using genes of the C_1 metabolism and isolation of the dominant bacteria using dedicated enrichment procedures.

The high diversity of the 16S rRNA genes in the clones was supported by the very diverse morphology observed in the TEM pictures. Recent developments to use gold labelled rRNA probes in thin sections of bacteria would enable the coupling of morphology and identity of the microorganisms (Gerard *et al*., 2005; Kenzaka *et al*., 2005) and would be a very promising tool in analyzing the microbial ecology.

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chapter 5

a microbial consortium couples anaerobic methane oxidation to denitrification

abstract

Modern agriculture has accelerated biological methane and nitrogen cycling on a global scale (Frankenberg *et al*., 2005; Galloway *et al*., 2004). Freshwater sediments often receive increased downward fluxes of nitrate from agricultural run-off and upward fluxes of methane generated by anaerobic decomposition (Conrad, 1996). In theory prokaryotes should be capable of using nitrate to oxidize methane anaerobically but such organisms have never been observed in nature nor isolated in the laboratory (Knowles, 2005; Shima & Thauer, 2005; Strous & Jetten, 2004; Valentine, 2002; Mason, 1977). Microbial oxidation of methane is thus believed to proceed only with oxygen or sulphate (Orphan *et al*., 2002; Boetius *et al*., 2000). Here we show that the direct, anaerobic oxidation of methane coupled to denitrification of nitrate is possible. A microbial consortium, enriched from anoxic sediments, oxidized methane to carbon dioxide coupled to denitrification in the complete absence of oxygen. The consortium consisted of two microorganisms, a bacterium representing a phylum without any cultured species and an archaeon distantly related to marine methanotrophic archaea. The detection of relatives of these prokaryotes in different freshwater ecosystems worldwide (Kasai *et al*., 2005; Koizumi *et al*., 2003; Bakermans & Madsen, 2002; Stein *et al*., 2001) indicates that the here presented reaction may make a substantial contribution to the biological methane and nitrogen cycles.

methods

sampling

Samples for inoculation were taken from the sediment of the Twentekanaal (52° 11' 04" N and 6° 24' 40" E, The Netherlands). Samples were collected from the top 15 cm of the sediment at 1 m water depth. At the time of sampling the methane concentration at 15 cm sediment depth was 0.8 mM. The nitrate concentration in the water column was 0.1 mM.

cultivation

The sediments samples of 6 sampling points were mixed and 1.0 liter was used as source of biomass in a sequencing batch reactor (SBR). The enrichment culture was performed according to the sequencing batch bioreactor principle1. The SBR was maintained in a 2 l (height, 0.22 m, diameter 0.125 m) vessel without baffles. The vessel was stirred at 350-500 rpm (6-bladed turbine stirrer, diameter one third of the vessel diameter). The temperature was 25°C. As the sole carbon and energy source, CH_4/CO_2 (95/5 % vol.) was added continuously by sparging (10 ml·min^{-1}). The oxygen concentration was monitored using an O_2-electrode and periodically the absence of oxygen from the headspace of the

culture was verified by GC. The CO_2 present in the gas was sufficient to buffer the solution and to keep the pH between 7.0 and 7.5. The SBR was filled continuously with mineral medium containing nitrate, nitrite, and bicarbonate (see below) at 0.2 ml·min^{-1} over 11.5 hours. After the filling period, the stirrer, influent and gas supply were stopped for 15 minutes to allow the aggregates to settle. In the remaining 15 minutes of the total cycle, the effluent pump drew off part of the liquid. To prevent entry of air and loss of anaerobiosis during the drawing-off, effluent gas from the culture was stored in a separate 10-liter flask that was flushed continuously with helium (20 ml·min^{-1}). The minimum liquid volume, after the liquid was drawn off, was 1.5 l. The maximum volume, at the end of the filling period, was 1.65 l. The composition of the mineral medium was (g·l^{-1}): $KHCO_3$ 1.25, KaH_2PO_4 0.05, $CaCl_2.2H_2O$ 0.3, $MgSO_4$. $7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625, trace elements solution 1.25 ml·l-1. $NaNO_2$ and $NaNO_3$ were added as specified in the results section. The trace element solution contained (g·l-1): EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4$ 0.25, $(NH_4)_6MoO_{24}.4H_2O$ 0.22, $NiCl_2$. $6H_2O$ 0.19, SeO_4 0.067, H_3BO_3 0.014, $Na_2WO_4.2H_2O$ 0.050. The separate components of the medium were autoclaved at 120°C. To adjust the pH and prevent entry of oxygen via the liquid medium, it was flushed continuously with Ar/CO_2 (95/5% vol.). The nitrate concentration was maintained above 3 mM at all times to prevent the occurrence of sulphate reduction.

analytical methods

Dinitrogen gas, oxygen and methane were analysed with a HP Agilent 6890 Series gas chromatograph equipped with a thermal conductivity detector and a Porapak Q column or with a HP 5890 gas chromatograph equipped with a flame ionization detector and a Porapak Q column. Nitrite and nitrate were measured with high performance liquid chromatography (HPLC). Liquid samples were centrifuged and 10 µl from the supernatant was injected with a Hewlett Packard 1050 Series autosampler. A sodium hydroxide solution was used as the liquid phase at a flow rate of 2 ml·min^{-1}. Nitrite and nitrate were eluted by a gradient of sodium hydroxide from 1 mM to 15 mM in 9 minutes. Separations were performed on a 4x250 mm Ionpac AS11-HC (Dionex, UK) column at 30°C. Anions were detected using a CD25 conductivity detector (Dionex UK). Total protein of 1 ml culture aliquots, stored at -20°C, was measured with the BCA™ Protein Assay Reagent from Pierce (USA.).

calculations

The values of the Gibbs energy changes reported with eq. 1 and 2 were calculated for a temperature of 25°C, pH 7 and for substrate concentrations of 0.1 mM. The affinity constant of a microbial conversion is the substrate concentration at which the conversion rate is half of the maximum conversion rate. In this case the affinity constant for methane was estimated from the slope of the methane consumption in Figure 2.

methane incorporation and lipid analysis

60 ml aliquots of the enrichment culture were anaerobically transferred to 120-ml serum bottles with an atmosphere of 90% argon and 10% ^{13}C-labelled methane. The bottles were incubated on a shaker at 30°C and sacrificed for lipid analysis after 3 and 6 days. Lipids were ultrasonically extracted after freeze drying and analysed by gas chromatography/mass spectrometry and isotope ratio gas chromatography mass spectrometry as described previously (Raghoebarsing *et al*., 2005). $^{13}CO_2$ as the end product of AOM was measured on a GC-Isotope Ratio Mass Spectrometry (ThermoFinnigan Delta Plus).

16S rRNA gene sequence analysis and FISH.

Chromosomal DNA from 1 ml reactor biomass, was isolated and used as template for PCR amplification of 16S rRNA genes. PCR was performed with general bacterial primers (616F & 630R), (Raghoebarsing *et al*., 2005), and general archaeal primers (AR20F & AR958R), (Hinrichs *et al*., 1999). Cloning, sequencing and phylogenetic analysis were performed as described previously (Raghoebarsing *et al*., 2005). Based on the bacterial and archaeal 16S rRNA gene sequences new oligonucleotide probes were designed. The bacterial probes were S-*-DBACT-0193-a-A-18 (5'-CGC TCG CCC CCT TTG GTC-3'), S-*-DBACT-0447-a-A-18 (5'- CGC CGC CAA GTC ATT CGT-3'), S-*-DBACT-1027-a-A-18 (5'- TCT CCA CGC TCC CTT GCG-3') and the archaeal probe was S-*-DARCH- 0872-a-A-18 (5'-GGC TCC ACC CGT TGT AGT-3'). We also used the general archaeal probe S-D-ARCH-0915-a-A-20, the general bacteria probe EUBmix and probe DSS658 for sulphate reducers (Boetius *et al*., 2000). FISH was performed as described previously (Raghoebarsing *et al*., 2005). Formamide concentrations used in the FISH experiments varied between 20% and 40%.

results & discussion

The global biogeochemical cycles are mainly driven by microorganisms feeding on one-carbon compounds such as methane or carbon dioxide. Each step in the element cycles is catalysed by a specific group of microorganisms. These may or may not be evolutionary related but they share the same lifestyle and so form an "ecological guild". Thermodynamic calculations show that most of these guilds have already been discovered (Figure 1), but the microorganisms that couple the anaerobic oxidation of methane (AOM) to denitrification (eq. 1 and 2) are considered missing in nature (Knowles, 2005; Shima & Thauer, 2005; Strous & Jetten, 2004; Valentine, 2002; Mason, 1977).

$5\ CH_4 + 8\ NO_3^- + 8\ H^+ \rightarrow 5\ CO_2 + 4\ N_2 + 14\ H_2O\ (\Delta G^{0'} = -464\ kJ \cdot mol^{-1}\ CH_4)$ (eq. 1)

$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O\ (\Delta G^{0'} = -673\ kJ \cdot mol^{-1}\ CH_4)$ (eq. 2)

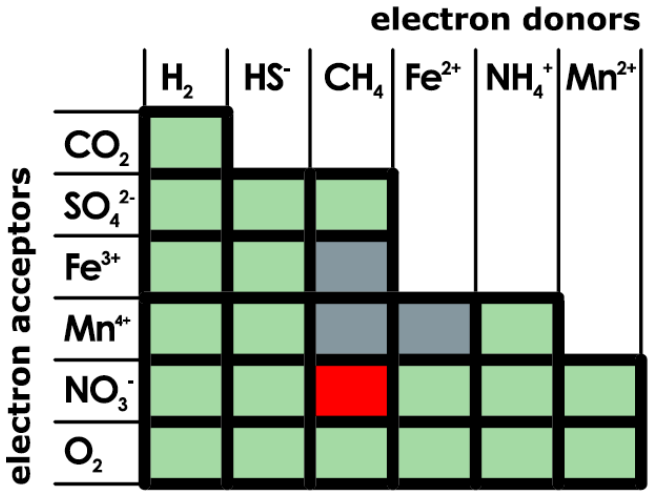

Figure 1: Contributions of one-carbon microorganisms to biogeochemical cycling. Each step in the element cycles is a redox reaction defined by an electron-donor and acceptor pair catalysed by a specific ecological guild of microorganisms. The figure indicates the steps in the element cycles that are known (green) and those that are still unknown but thermodynamically possible (grey). The anaerobic oxidation of methane (AOM) coupled to denitrification is indicated in red and is a possible link between the carbon and nitrogen cycles. (see page 103 for color figure).

Since AOM coupled to denitrification is possible in theory, both thermodynamically and biochemically (via reverse methanogenesis) (Hallam *et al.*, 2004; Krüger *et al.*, 2003), such microorganisms may in fact exist and consequently our understanding of biogeochemical methane cycling may be incomplete. The lack of experimental evidence for the occurrence of AOM coupled to denitrification is perhaps not surprising because the process would be

expected to occur close to the oxic/anoxic interface. This interface is generally characterized by steep gradients, occurring within millimeters, masking the process from geochemical detection. Furthermore, the laboratory enrichment of the responsible microorganisms could be difficult because of their potentially very slow growth (Strous *et al*., 2002).

Here we report on the successful enrichment of consortia of microorganisms capable of AOM coupled to denitrification. Anoxic sediment of a canal (Twentekanaal, the Netherlands) was used as the inoculum for the enrichment culture. This canal contained nitrate at concentrations up to 1 mM and the sediment was saturated with methane, typical for freshwater habitats receiving agricultural run-off. A one-litre sample from the upper layer of the sediment was incubated anoxically in the laboratory. Methane was supplied as the only electron donor and mineral medium containing nitrate, nitrite, bicarbonate and trace elements was supplied and removed continuously. During 16 months of incubation the influent nitrite concentration was gradually increased to 6 mM, while the actual concentration in the culture medium remained around 0.1 mM, indicating growth of a microbial population consuming nitrite. Some nitrate (< 1 mM) was also consumed. Methane consumption could not yet be observed experimentally because it was supplied in large excess and any potential conversion remained within the error margin for the CH_4 analysis.

To measure methane consumption, the media and methane supply were stopped and excess methane was removed by flushing with helium gas. The consumption of methane, nitrite and nitrate was now apparent and dinitrogen gas evolved (Figure 2). Nitrite and nitrate together accounted for all of the produced dinitrogen gas. However, the total denitrification rate (28.8±2 µmol $N_2 \cdot h^{-1}$) was not completely accounted for by the methane oxidation (13.4±1 µmol $CH_4 \cdot h^{-1}$) according to equations (1) and (2). Control experiments indicated that this difference was caused by the oxidation of organic compounds from the inoculum or the mineral medium. In these control experiments the denitrification rate in the absence of methane was 5.5±0.5 µmol $N_2 \cdot h^{-1}$ and upon methane addition increased to 21.5±2 µmol $N_2 \cdot h^{-1}$. Since methane itself was consumed with 22.0±2 µmol $CH_4 \cdot h^{-1}$, the stoichiometry of AOM was almost completely consistent with the above equations.

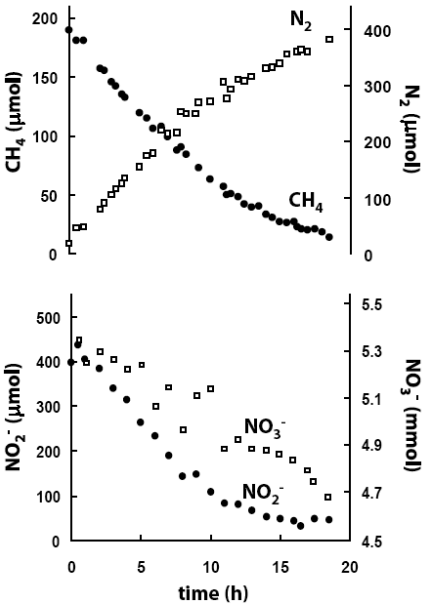

Figure 2: AOM is coupled to denitrification of nitrite by the enrichment culture after 16 months of enrichment. The total amounts of methane, dinitrogen gas, nitrate and nitrite present in the culture vessel are indicated. The initial concentrations of these compounds in the culture liquid were 6.0 and 0.30 µM, 3.6 and 0.24 mM, respectively. During this experiment, the total amount of protein in the enrichment culture was 100 mg.

The enrichment culture preferred nitrite to nitrate as the substrate for denitrification; in experiments where nitrite was depleted in the presence of both methane and nitrate, AOM ceased. It only resumed with the addition of nitrite after 2 h. However, after longer incubation in the absence of nitrite (10-20 h) AOM was also restored and was then coupled to nitrate consumption. Apparently, the enrichment culture could adapt to nitrate and both nitrite and nitrate were suitable substrates for AOM (equations (1) and (2). The apparent affinity constant for methane was less than 0.6 µM (Figure 1) indicating that the affinity for methane was very high. The methane affinity of sulphate dependent AOM is four orders of magnitude lower (affinity constant > 16 mM) (Nauhaus *et al*., 2005). The addition of sulphate to the enrichment culture (2 mM) neither stimulated nor inhibited AOM.

Together, these experiments show unambiguously that methane was oxidized anaerobically, and that this oxidation was coupled to denitrification. The participation of oxygen can be fully excluded; first, oxygen was measured continuously in the culture liquid and periodically in the headspace but was never detected (detection limit 80 p.p.m). Second, all the detected dinitrogen gas in the headspace of the culture was accounted for by the consumption of nitrite and nitrate. If air had leaked into the culture, more dinitrogen gas would have been detected in the headspace than was predicted by the reaction stoichiometry. Third, the stoichiometry of methane consumption coupled to

denitrification was in good agreement with equations (1) and (2). Had oxygen been involved, much less nitrite or nitrate would have been consumed per mol of methane. Finally, no methane was consumed as the enrichment culture adapted from nitrite to nitrate (see above).

We investigated the incorporation of methane into microbial biomass by analysing the membrane lipids of the enrichment culture. We detected a single archaeal and several bacterial biomarkers (Table 1). The archaeal biomarker sn2-hydroxyarchaeol, found in methanogens (Koga *et al.*, 1998) and in ANME-2 methanotrophic archaea (Blumenberg *et al.*, 2004), was the only biomarker substantially depleted in ^{13}C compared to methane.

Table 1: Incorporation of methane into the bacterial and archaeal biomarkers of the enrichment culture

			Enrichment culture (t = 16 months)	After labelling with ^{13}C methane	
				3 days	*6 days*
Compounds	*Amount (%)*	*Origin*[b]	*$\delta^{13}C$ (‰ vs VPDB)*	*$\delta^{13}C$ (‰ vs VPDB)*	*$\delta^{13}C$ (‰ vs VPDB)*
Methane	-		-40.7		
Bicarbonate	-		-24.6		
$C_{14:0}$ FA[a]	0.8	B	-26.1	+2300	+4400
Iso-$C_{15:0}$ FA	2.3	B	-31.0	+125	+189
$C_{16:1}$ (^{9}) FA	6.8	B	-43.7	+4300	-[c]
$C_{16:0}$ FA	11.4	B	-30.5	+370	+580
10-me-$C_{16:1}$ (^{7}) FA	5.5	B	-37.9	-[d]	-[d]
10-me-$C_{16:0}$ FA	28.9	B	-38.5	-36.4	-36.5
$C_{18:0}$ FA	2.8	B	-31.4	+310	+380
$C_{18:1}$ (^{11}+ ^{10}) FA	14.3	B	-35.8	+900	-[c]
$C_{19\ cycloprop.}$ FA	12.7	B	-38.0	+290	+420
Diplopterol	4.1	B	-46.6	+380	-[c]
sn2-hydroxyarchaeol	2.2	A	-67	-76	-49

VDPB, Vienna Pee-Dee Belemnite

The table shows the relative abundances and stable carbon isotopic composition of the major lipids of the enrichment culture and the incorporation of ^{13}C into these compounds after 3 and 6 days of incubation with ^{13}C labelled methane ($\delta^{13}C = [(^{13}C/^{12}C sample/(^{13}C/^{12}C standard]-1)$. For the archaeal compound from the enrichment culture, the large depletion in ^{13}C indicated that its carbon was derived from (^{13}C-depleted) methane. The minor but significant incorporation of ^{13}C into this compound during labelling ($\Delta\delta^{13}C$ = -49 minus -67 = +18‰) suggested slow growth. [a] FA = fatty acid; [b] B = bacteria, A = archaea; [c] $\delta^{13}C$ after 6 days was not determined for some lipids already substantially enriched in ^{13}C after 3 days; [d] $\delta^{13}C$ could not be determined due to low abundance of the compound and co-elution.

This indicated that the carbon in this compound originated from methane. When ^{13}C labelled methane was now supplied to the culture, incorporation into sn2-hydroxyarchaeol was also observed after 6 days. Interestingly, the bacterial biomarkers were labelled more rapidly and substantially (Table 1). These results are comparable to those obtained for a similar ^{13}C labelling experiment with a consortium performing sulphate-dependent AOM (Blumenberg *et al.*, 2005), where rapid and substantial incorporation of ^{13}C-labelled methane was noted in the lipids of the sulphate reducing bacteria whilst only after prolonged incubation (>300 days) minor incorporation of ^{13}C was observed in the archaeal lipids. Our biomarker data thus indicate that a consortium consisting of an archaeon and a bacterium is responsible for AOM coupled to denitrification.

To determine the phylogenetic identity of the members of this consortium, genomic DNA was isolated from the biomass in the enrichment culture and bacterial and archaeal 16S rRNA gene libraries were constructed. Sequence analysis of the bacterial clone library showed one dominant group of sequences clustering inside a subdivision without any cultivated species (Figure 3a). This subdivision was distant from all other bacterial subdivisions (sequence identity less than 85%). Similar sequences of this subdivision were retrieved previously from the denitrifying zone of sediments of Lake Biwa, Japan (Koizumi *et al.*, 2003) and from contaminated groundwater in the United States (Bakermans & Madsen, 2002). The archaeal clone library contained a single sequence which was only distantly related to the AOM archaea of group 2 (ANME-2, 86-87% identity) (Orphan *et al.*, 2002; Knittel *et al.*, 2005) and cultivated methanogens, (86-88% identity, Figure 3b). The highest similarity was found with archaeal clone sequences obtained from freshwater sediments from Lake Michigan in the United States (Stein *et al.*, 2001) and contaminated soils in Japan (Kasai *et al.*, 2005).

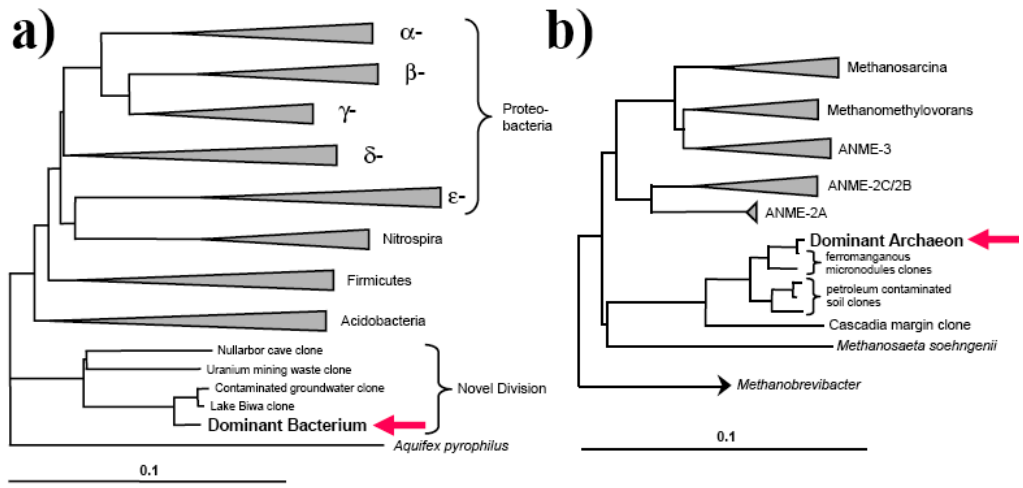

Figure 3: Phylogeny of the archaeal and bacterial members of the consortium mediating AOM coupled to denitrification. Consensus trees of the dominant (a) bacterial and (b) archaeal 16S rRNA sequences of the enrichment culture. Scale bars = 10 base substitutions per 100 bases. The 16S rRNA gene sequences were deposited at GenBank under accession numbers DQ369741 (archaeal sequence) and DQ369742 (bacterial sequence).

We used both the bacterial and archaeal 16S rRNA gene sequences to design specific probes for fluorescence *in situ* hybridization (FISH). Samples collected during the 16 month period of enrichment were now hybridized with these probes. In samples from the first three months, only occasional, single cells tested positive. Over time, both the bacterium and the archaeon became more and more enriched until they became the dominant microorganisms in the culture. After 16 months, approximately 10% of the DAPI-stained cells consisted of Archaea, all of which hybridized with the specific probe targeting the dominant archaeal sequence. The remainder of the culture consisted of bacteria, of which approximately 80% hybridized with the three specific probes targeting the dominant bacterial sequence (Figure 4). As shown in Figure 4, the archaea were generally present as clusters inside a matrix of bacterial cells. The ratio of bacterial to archaeal cells (approximately 8:1) was different from the ratio reported for sulphate dependent AOM (approximately 2:1), (Nauhaus *et al.*, 2005). This difference might be explained by the higher energy yield of denitrification compared to sulphate reduction. Alpha, beta and *gammaproteobacteria* together made up <5% of the community. The sulphate reducers known to be involved in sulphate dependent AOM (Boetius *et al.*, 2000) were not detected consistent with the observation that sulphate was not converted in the culture.

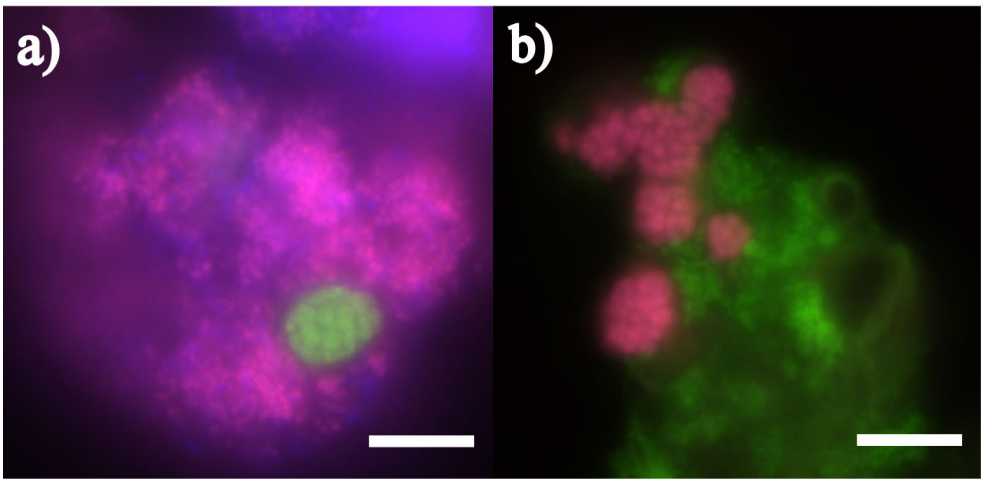

Figure 4: Fluorescence *in situ* detection of the archaeal and bacterial members of the consortium (a) Epifluorescence micrograph after hybridization with the general bacterial probe EUBmix (blue, Cy5), the specific bacterial probe DBACT-193 (red, Cy3) and the specific archaeal probe DARCH-872 (green, FLUOS). The bacterial partner is pink, as it hybridizes with both the general and specific bacterial probes. (b) Epifluorescence micrograph after hybridization with the general archaeal probe ARCH915 (blue, Cy5), the specific archaeal probe DARCH-872 (red, Cy3) and the general bacterial probe EUBmix (green, FLUOS). The archaeal partner is pink because it hybridizes with both the general and specific archaeal probes. Scale bars, 5 µm. See Methods for specifications of probes. (see page 103 for color figure).

The nitrite dependent AOM rate was 140 µmol-CH_4 g-protein^{-1} hour^{-1} (Figure 2), corresponding to approximately 0.4 fmol-CH_4 cell^{-1} day^{-1} for the archaea in our enrichment culture. For sulphate-dependent AOM, a similar rate was reported for the archaeal partner (0.7 fmol-CH_4 cell^{-1} day^{-1}), (Nauhaus *et al.*, 2005). This indicates that for AOM coupled to denitrification, the archaeal growth rate could be extremely low, with a doubling time in the order of several weeks, consistent with the long duration of the enrichment procedure. To our knowledge this is the first report of archaeal AOM coupled to bacterial denitrification. In the 1970s, Mason (1977) discredited earlier studies that pure cultures of methanotrophic bacteria were able to denitrify with methane as the sole carbon and energy source. Instead, it was established experimentally that methanotrophs can oxidize methane aerobically to methanol or acetate at low oxygen concentrations and that the methanol or acetate can subsequently be used to drive denitrification (Islas-Lima *et al.*, 2004; Waki *et al.*, 2002; Eisentraeger *et al.*, 2001; Costa *et al.*, 2000). Thus, the here presented reaction defines a newly discovered microbial guild and its potential contribution to biogeochemical cycling has so far been overlooked. The recovery of related 16S rRNA gene sequences from different habitats and locations (Kasai *et al.*, 2005; Koizumi *et al.*, 2003; Bakermans & Madsen, 2002; Stein *et al.*, 2001) indicates that the process may contribute significantly to methane oxidation. Potentially it could counteract worldwide

increases in methane production associated with intensive agriculture. With the biomarkers and probes for the responsible microorganisms now available, this possibility can be assessed.

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curriculum vitae

Ashna Anjana Raghoebarsing werd op 4 januari 1975 geboren te Paramaribo (Suriname) en kwam op haar zestiende naar Nederland. In 1994 behaalde zij haar HAVO diploma in Den Haag. Daarna studeerde zij Milieu Chemie aan het Hoger Laboratorium Onderwijs te Delft. Zij liep stage bij de Technische Universiteit Delft (Milieu Microbiologie), alvorens in 1998 af te studeren. Hierna ging zij Biologie studeren aan de Universiteit Leiden. Tijdens deze studie liep zij stages bij Moleculaire Genetica (UL) en John Innes Centre (Norwich, UK), en slaagde ze in 2000 voor haar doctoraalexamen. In 2001 startte zij haar promotieonderzoek bij de afdeling Ecologische Microbiologie aan de Radboud Universiteit Nijmegen. Het onderzoek, beschreven in dit proefschrift, werd uitgevoerd onder begeleiding van Dr. Ir. Marc Strous en Prof. Dr. Ir. Mike Jetten. Sinds 1 mei 2006 is Ashna werkzaam als Projectadviseur Energie en Milieu bij de afdeling EG-Liaison van SenterNovem in Den Haag.

publication list

Raghoebarsing, A.A., Smolders, A.J.P., Schmid, M.C., Rijpstra, W.I.C., Wolters-Arts, M., Derksen, J., Jetten, M.S.M., Schouten, S., Sinninghe Damsté, J.S., Lamers, L.P.M., Roelofs, J.G.M., Op den Camp, H.J.M. & Strous, M. (2005). Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. *Nature* **436**: 1153-1156. [chapter 2]

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Raghoebarsing, A.A., Schmid, M.C., Verwegen, K., Welboren, W., Smolders, A.J., Op den Camp, H.J., Jetten, M.S. & Strous, M. Microbial diversity in two *Sphagnum* peat bogs. [chapter 4, submitted]

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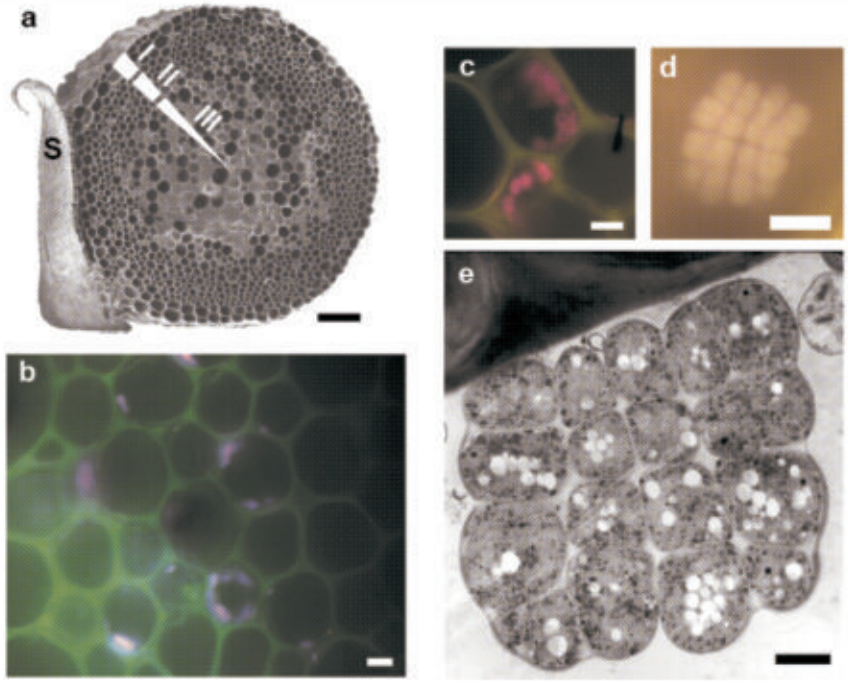
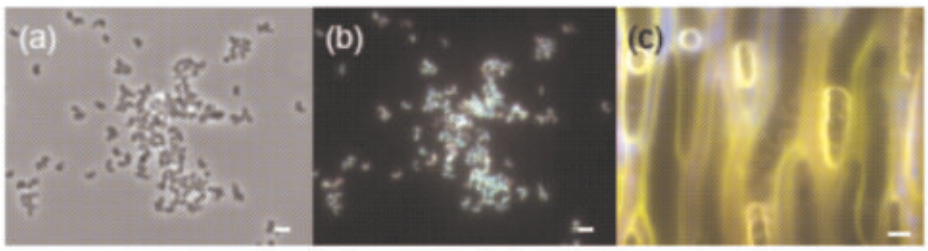
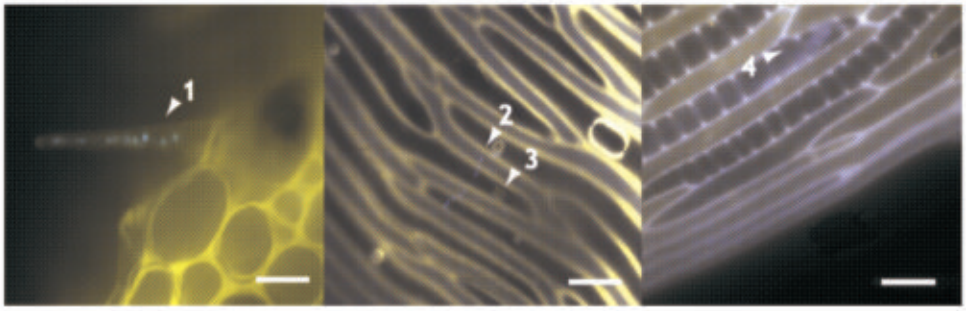
color figures



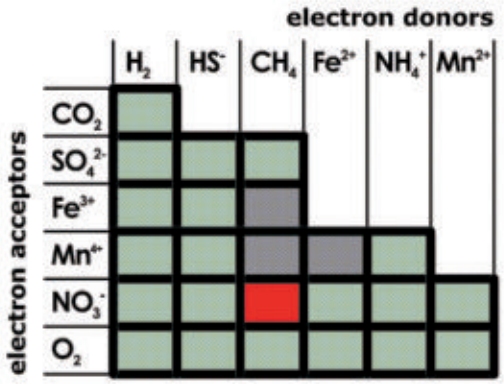
Chapter 2, Figure 2: *In situ* detection of the new methanotroph in *S. cuspidatum* with fluorescently labelled rRNA-targeted oligonucleotide probes. (a) Cryo-scanning electron micrograph of a stem cross section. S, stem leaf; I, outer cortex; II, internal cylinder; III, inner pith. Scale bar 100 µm. (b), (c) Epifluorescence micrographs of the new methanotroph (purple or pink cells) in the outer cortex of *Sphagnum* stems, after a double hybridization with the specific probe 18ALF1437 and the general probe EUB. Scale bars 10 and 5 µm. (d) Dense, geometric clusters of the same bacterium on a stem leaf, after a triple hybridization with the specific probe18ALF1437, the general probe EUB and probe ALF968 (specific for *alphaproteobacteria*). Scale bar 5 µm. (e) Transmission electron micrograph of a geometric cluster closely attached to a stem leaf. Scale bar 1 µm.



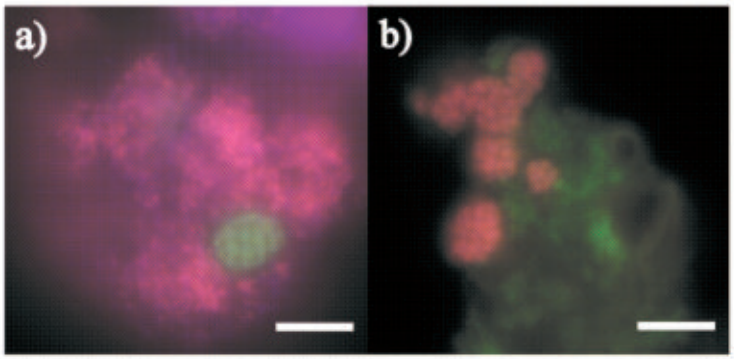
Chapter 3, Figure 4: (a) Fluorescence *in situ* hybridisation of strain 29 with the newly developed specific rRNA-targeted oligonucleotide probe (labeled with Cy3, red), the Bacteria-specific EUB probe mix (Fluos, green) and ALF968 (specific for *alphaproteobacteria*, Cy5, blue). Strain 29 cells appear white, because of overlapping labels; bar, 2 µm. (b) FISH on a stemleave of *S. cuspidatum* hybridised with the same probes as in (a), bar, 5 µm.



Chapter 4, Figure 3: *In situ* detection of *Planctomycetales* related microorganisms in *S. cuspidatum*. Epifluorescence micrographs of the hybridization of the two specific probes and the general bacterial probe (a) with the stem. Coccoid cells (arrow 1) hybridizing with all tree probes are colored light blue, (b) with a stem leaf. Chain structures cells hybridizing with all tree probes are colored yellow (arrow 2) and sarcina-like cells hybridizing with the bacterial probe (arrow 3), (c) also with a stem leaf. Coccoid cells inside the hyaline cells hybridizing with all tree probes are colored light-blue (arrow 4). Scale bar, 10 µm.



Chapter 5, Figure 1: Contributions of one-carbon microorganisms to biogeochemical cycling. Each step in the element cycles is a redox reaction defined by an electron-donor and acceptor pair catalysed by a specific ecological guild of microorganisms. The figure indicates the steps in the element cycles that are known (green) and those that are still unknown but thermodynamically possible (grey). The anaerobic oxidation of methane (AOM) coupled to denitrification is indicated in red and is a possible link between the carbon and nitrogen cycles.



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